Novel fusion protein-expressing lentiviral vectors ameliorate collagen induced arthritis

Submitted for the degree of

doctor of philosophy

by

Eliot Ward

for elaine

Contents

2 Materials and methods

3 Generation and expression of fusion constructs

4 *In vitro* characterization of fusion proteins

5 Vaccination with vCII-LAMP is protective in CIA

6 Further applications

7 General discussion

Statement of work completed by the candidate

I, Eliot Michael Ward, declare that all the work presented in this thesis is my own, with the following exceptions:

Vaccination of DBA/1 mice with lentiviral vectors prepared my me was performed by Inger Gjertsson at the Department of Rheumatology at the University of Gothenburg, Sweden. Subsequent immunisation with rat collagen II, termination of the *in vivo* experiment and subsequent stimulation of harvested splenocytes and lymph node cells were also performed by Inger Gjertsson.

All DNA manipulation, virus preparation and titre and *in vitro* experiments were performed by myself. Additionally, during the first *in vivo* experiment, I spent time with Inger Gjertsson at the Department of Rheumatology, University of Gothenburg where I took blood samples and learned to score the mice for physical signs of arthritis.

Publications emanating from this work

Ward, E. M., Chan, E., Gustafsson, K., & Jayasinghe, S. N. Combining bio-electrospray with gene therapy: a novel biotechnique for the delivery of genetic material *via* living cells. Analyst . 27-2-2010.

Wu, Y., Wu, W., Wong, W. M., Ward, E., Thrasher, A. J., Goldblatt, D., Osman, M., Digard, P., Canaday, D. H., & Gustafsson, K. 2009, "Human gamma delta T cells: a lymphoid lineage cell capable of professional phagocytosis", *J Immunol*, vol. 183, no. 9, pp. 5622-5629.

Abstract

Collagen induced arthritis (CIA) is a mouse model of autoimmunity that closely resembles human rheumatoid arthritis (RA), a debilitating disease with no cure. This study has been undertaken to generate antigen-specific tolerance to an autoantigen implicated in RA and immunodominant in animal models. Gene therapy protocols for RA that have gone to clinical trial have been designed to drive expression of therapeutic molecules at the site of inflammation, but not to modulate the immune response to key autoantigens.

This study has shown that lentiviral vectors (*lvv*) expressing fusion proteins (FP-*lvv*) confer antigen-specific tolerance in CIA. Fusion proteins comprised of an endosomal-targeting domain coupled to the immunodominant $CII_{259-273}$ peptide and an eGFP tag, were expressed in APCs. Confocal microscopy revealed substantial colocalisation with endosomes and lysosomes. Expression of the fusion proteins in APCs resulted in MHCII-presentation of the immunodominant $CII_{259-273}$ peptide to $CII_{259-273}$-reactive CD4+ T cell hybridomas. Furthermore, co-transduction with a *lvv* expressing the enzyme lysyl-hydroxylase 3 enhanced glycosylation of the expressed CII construct.

Administering mice *iv* with FP-*lvv* one month prior to disease induction reduces by half the arthritic score during the first two weeks of clinical symptoms in CIA, providing partial but significant protection. The use of suitable controls showed that this effect is antigen-specific and measurements of α-CII IgG show a significantly lower titre in treated animals.

This study provides evidence that *lvv*-mediated MHCII-presentation can be tolerogenic and hence, this approach could form an important part of future treatments for autoimmune diseases.

List of abbreviations

3T3-I-Aq	3T3 fibroblasts stably expressing the MHCII-I-Aq molecule
aa	Amino acid
AAV	Adeno-associated virus
ADA	Adenosine deaminase
AIA	Antigen-induced arthritis
AICD	Activation-induced cell death
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APL	Altered peptide ligand
BCR	B cell receptor
BES	Bio-electrospray
CA	Capsid
CAIA	α-Collagen antibody induced arthritis
cAMP	Cyclic adenosine monophosphate
CCR5	Chemokine C-C motif receptor 5
CD	Cluster of differentiation
CE	Cell electrospray
CFA	Complete Freund's adjuvant
CI	Collagen type I
CIA	Collagen induced arthritis
CII	Collagen type II
CII-DM	$CII_{259-273}$-containing H2-DM-domain-targeted fusion protein
CII-LAMP	$CII_{259-273}$-containing LAMP-1-domain-targeted fusion protein
CLIP	Class II-associated invariant chain peptide
Con A	Concanavalin A

CPM	Counts per minute
cPPT	Central polypurine tract
CTE	Constitutive transport element
CTL	Cytotoxic lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTS	central termination sequence
CXCR4	CXC chemokine receptor
CypA	cyclophilin A
DC	Dendritic cell
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalitis
EEA-1	Early Endosome-associated antigen
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FlAsH	Bis-arsenical fluorescein derivative
FP-*lvv*	Fusion protein-expressing lentiviral vector
Gal(β1-O)	β-galactopyranose monosaccharide
Glc(α1-2)Gal(β1-O)	α-glucosyl-1,2-β-galactopyranose
HIV	Human immunodeficiency virus
HSV-tk	herpes simplex virus-thymidine kinase
IFA	Incomplete Freund's adjuvant
IFN-γ	Interferon-γ
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
IL-23R	Interleukin-23 receptor

IL-2ss	Interleukin-2 signal sequence
IN	Integrase
ip	Intra-peritoneal
iTregs	Induced regulatory T cells
IUPAC	International Union of Pure and Applied Chemistry
iv	Intravenous
LAMP-1	Lysosome-associated membrane protein
LCA	Leber's Congenital Amaurosis
LH3	Lysyl-hydroxylase-3
LMO2	LIM domain only 2
Lss	LAMP-1 signal sequence
LTR	Long terminal repeat
lvv	Lentiviral vector
MA	Matrix protein
MEC	Medullary epithelial cell
MHCII	Major histocompatibility complex class II
MIIC	MHCII-containing compartments
MLV	Murine leukaemia virus
MOG	Myelin oligodendrocyte glycoprotein
MOG-LAMP	MOG_{79-90}-containing LAMP-1-domain-targeted fusion protein
MOI	Multiplicity of infection
MP	Matrix peptide
mRNA	Messenger ribosenucleic acid
МΦ	Macrophage
n/s	Not significant
NC	Nucleocapsid protein
NK	Natural killer
nTregs	Natural T-regulatory cells
OTC	Ornithine transcarbamylase

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBS	Primer binding site
PCR	Polymerase chain reaction
PEI	Polyethylenemine
PEO	Polyethylene oxide
PFA	Paraformaldehyde
PIC	Pre-integration complex
PPT	Polypurine tract
PRR	Pattern recognition receptors
PTA	Peripheral tissue antigen
qPCR	Quantitative polymerase chain reaction
R	Repeat region
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RNase H	Ribonuclease-hybrid
RORγ	RAR-related orphan receptor γ
RT	Room temperature
RT	Reverse transcriptase
RTC	Reverse transcription complex
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
sffv	Spleen focus forming virus
SICW	*sffv*-Ii-CII$_{259-273}$-WPRE *lvv*
SIN	Self inactivating
SRP	Signal recognition particle
ssRNA	Single-stranded RNA
-sssDNA	Minus-strand strong stop DNA
T-ALL	T-cell acute lymphoblastic leukaemia

TAR	Trans-activation response element
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th1/2	Helper T cell type 1/2
TLR	Toll like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
U3/5	Unique 3'/5' region
vCII-DM	CII-DM-expressing *lvv*
vCII-LAMP	CII-LAMP-expressing *lvv*
vMOG-LAMP	MOG-LAMP-expressing *lvv*
VSV-G	Vesicular stomatis virus glycoprotein
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
X-CGD	X-linked chronic granulomatous disease
γc	Common γ-chain

Chapter 1. Introduction

1 Introduction

1 *Tolerance and immunity*

The immune system serves to protect an organism from disease. Multiple mechanisms act to detect and react to pathogens as diverse as foreign cells, infected or altered self cells, virus particles and other harmful particles or molecules. Classically divided into innate and adaptive immunity, the interface between what was once regarded as two separate systems is now known to be critical in shaping the adaptive response. This project can be viewed as an investigation of how inducing an interaction between innate and adaptive systems can modulate subsequent immune reaction to a model antigen.

Phagocytic cells have an "innate" capacity to recognise and ingest harmful materials, and those endowed with antigen presenting abilities such as dendritic cells (DCs) and macrophages (MΦ) not only remove and digest the offending substance, but also present digested fragments to a particular subset of lymphocytes that constitute the "adaptive" arm, alerting them to the threat. It is at this interface between the innate and adaptive systems, where presented antigens prime the adaptive response that can largely determine the nature of the response to particular antigens.

Lymphocytes use antigen receptors to mediate adaptive immunity, and there is an almost infinite variety of antigen receptors, generated through random rearrangement of Ig genes in B lymphocytes and T cell receptor (TCR) genes in T lymphocytes. The ability to generate so vast a range of antigen receptors is crucial for the recognition of epitopes present in any immunological challenge, but because the generation of diversity in antigen receptors is random, many lymphocytes develop with antigen receptors that react against the host's own constituents. However, harmful immune reactions against the host are rare because another series

of mechanisms exist to tolerise the immune systems to host antigens, preventing autoimmunity (Goodnow et al. 2005).

This project has shown that by expressing chimeric proteins that traffic antigens to the endosomal pathway in antigen presenting cells (APCs) the adaptive system can be primed to respond to subsequent challenge from that particular antigen much less severely. Chimeric protein expression was driven by self inactivating (SIN) lentiviral vectors.

This study, therefore, shows that induction of antigen presentation using this method tolerises and may prove to have important applications in the treatment of autoimmune diseases.

1.1 *Tolerance induction in the healthy immune system*

In a 'normal' immune system, tolerance to self antigens and other innocuous, non-infectious environmental antigens is achieved by silencing the immune response in an antigen-specific manner. This, as discussed below, can be done centrally or in the periphery, by a number of overlapping mechanisms such as clonal deletion or desensitisation of autoreactive lymphocytes (Nossal 1994), or by immunological suppression by regulatory T cells (Treg cells) specific to the innocuous or self antigen (Kronenberg & Rudensky 2005). As there is no direct way to determine whether a particular molecule has originated from the host, the context in which the immune system encounters an antigen shapes the response and subsequent responses to that antigen.

1.1.1 *Central tolerance*

The first point at which self-reactivity can be silenced is during lymphocyte development that happens in the central lymphoid organs of the bone marrow and thymus, during which strongly reactive lymphocytes are deleted or altered so as not to respond aggressively to the stimulating antigen. In this way, the immune system uses the context of centralised antigen recognition as a marker of self. B cell

receptors react with antigens in their native form (eg folded peptide epitopes) wheras T cells react with antigens presented on Major Histocompatibility (MHC) molecules such as MHC class II (MHCII) (figure 1.1)

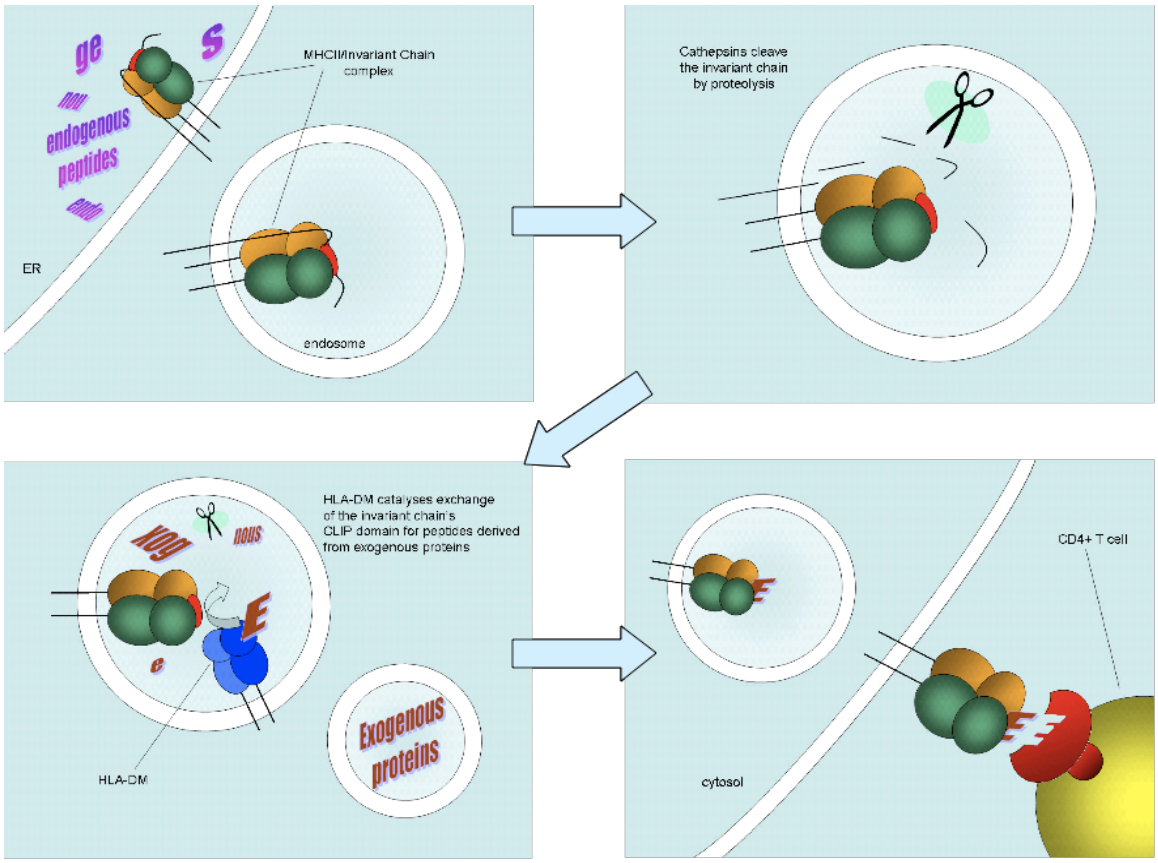


Figure 1.1 Antigen processing and presentation

MHCII molecules fold in the Endoplasmic Reticulum (ER) in complex with the Invariant Chain, Ii, which includes a CLIP domain that excludes other endogenous peptides in the ER from occupying the antigen-binding groove of MHCII (top left). The trimeric complex is trafficked to the lysosomes/late endosomes where cathepsins degrade the invariant chain (top right) and also captured antigens. HLA-DM (H2-DM in mice) is a chaperone molecule, structurally related to MHCII, which acts to facilitate CLIP exchange for processed antigen (above left). The MHCII-antigen complex then moves to the plasma membrane for presentation to CD4+ T cells (above right).

Both B and T cell progenitors originate in the bone marrow, but whilst important steps in the deletion or inactivation of autoreactive B cells occur in the bone marrow, T cell progenitors travel to the thymus to continue their development. Medullary epithelial cells (MECs) present peptides from a wide array of host-derived peripheral tissue antigens (PTAs), usually found only in specific tissues, on MHC molecules (Westerberg, Klein, & Snapper 2008). Interaction between the T-cell receptor and MHC-peptide complex is crucial for the positive selection and survival of the thymocyte (Fowlkes & Schweighoffer 1995) because this ensures that the mature T cell will recognise peptides presented on self-MHC molecules. However, T-cell clones that strongly react to MHC/self-peptide complexes are deleted (Kappler, Roehm, & Marrack 1987;Kisielow et al. 1988;Kishimoto & Sprent 1997) by induced apoptosis (clonal deletion) (Surh & Sprent 1994) or can be induced to develop down the lineage of regulatory T (Treg) cells (Itoh et al. 1999;Picca & Caton 2005) which will be discussed further below. The importance of central deletion of strongly reactive T cells in the thymus is illustrated when PTA expression is abrogated. Although its precise regulatory mechanism remains unclear, the autoimmune regulator gene *Aire* (Aaltonen et al. 1997;Nagamine et al. 1997), expressed at high levels in MECs (Anderson et al. 2002;Anderson et al. 2005), promotes ectopic expression of PTAs. Mutations in this gene lead to the rare condition autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). In *Aire*-knockout mice, the self-reactive lymphocytes escape clonal deletion and are able to egress from the thymus, migrating to the periphery (Anderson et al. 2005) resulting in multi-organ autoimmunity (Mathis & Benoist 2007). Hence, the ability of the immune system to recognise a variety of self-antigens in the context of central presentation is crucial for generating central tolerance.

As many as 75% of early immature B cells have been found to express self-reactive antibodies (Wardemann et al. 2003), but most of these are removed during B cell maturation. Developing autoreactive B cells are silenced within the bone

marrow by three known mechanisms (Meffre & Wardemann 2008). Strongly self-reactive B cells maturing in the bone marrow are either deleted (Nemazee & Burki 1989;Cornall, Goodnow, & Cyster 1999) or the self-reactive B-cell receptor (BCR) can be internalised and edited to replace the light chain with a version that is no longer self-reactive so as to escape clonal deletion (Tiegs, Russell, & Nemazee 1993;Halverson, Torres, & Pelanda 2004). This is thought to occur in at least 25% of developing B cells in normal individuals (Retter & Nemazee 1998;Casellas et al. 2001). Thirdly, B cells that chronically bind self-antigen may become anergic, defined as functional inactivation to further signals through their BCR (Hippen et al. 2005), a state initially described in 1988 (Goodnow et al. 1988). Finally, B cells can be inherently unresponsive due to ignorance of their autoantigen if the antigen is inaccessibly sequestered in a cellular compartment.

Clonal deletion, or conversion from potentially dangerous autoreactive lymphocytes into either non-autoreactive lymphocytes, or into immunosuppressive, autoreactive Treg cells, silences the immune response from cells with antigen receptors that bind strongly in the primary lymphoid organs; strong reactivity in the context of developing lymphocytes in the central lymphoid organs is used to centrally silence potential autoimmune reactions. Some self antigens, however, may not have access to developing lymphocytes (Lo et al. 1989) and these clonally ignorant lymphocytes may encounter their cognate autoantigens after leaving the central lymphoid organs. Additonally, some autoreactive lymphocytes escape the mechanisms for central tolerance (Bouneaud, Kourilsky, & Bousso 2000). There are a plethora of mechanisms in the periphery that prevent autoreactive lymphocytes from mounting a destructive response against the host, many of which are still being elucidated. Below is an overview of peripheral immune regulation, focussing on antigen recognition by CD4+ T cells that are pivotal in the deployment of the adaptive immune response. Again, the context in which the thymocyte encounters its cognate antigen determines the nature of any subsequent immune responses.

1.1.2 *CD4+ T cells are pivotal in the deployment of the adaptive immune response*

Tolerisation of CD4+ T cells is critical in avoiding autoimmune disease. Often termed helper T cells (Th cells), CD4+ T cells do not generally exert any direct cytotoxic action against pathogens but instead 'help' other arms of the immune system by activating other effector cells, inducing them to mount an appropriate response. The exceptions are regulatory CD4+ T cells (Tregs) that express the forkhead/winged-helix transcription factor FoxP3 (Hori, Nomura, & Sakaguchi 2003).

Th cells were historically divided into two classes namely Th1 and Th2 (Mosmann & Coffman 1989) according to their cytokine profile that is critical in activating a particular branch of the immune system. Antigen-specific MΦ activation by gamma-interferon (IFN-γ) producing CD4+ T cells (helper class 1, Th1) are required for the induction of antimicrobial mechanisms against many intracellular pathogens (Stout & Bottomly 1989), whilst interleukin-4 (IL-4) producing helper T cells (Th2) are required for B cell activation in most cases (Parker 1993). Even the cytotoxic response to infection, mediated by CD8+ cytotoxic T lymphocytes (CTLs) involves regulation from CD4+ T cells (Novy et al. 2007) and CD4+ T cell help is especially important in the generation and maintenance of memory CD8+ T cells (Ramsburg et al. 2007).

More recently a third group of effector CD4+ cells have been described that have been shown to be distinct from Th1/Th2, denoted Th17 cells that produce IL-17 (Aggarwal et al. 2003). IL-17 contributes to the pathogenesis of arthritis (Lubberts 2003) and it has been shown that IL-17-deficient mice are less susceptible to CIA, partly due to impaired CII-specific IgG2a production (Nakae et al. 2003). Th17 cells were initially thought to be induced by IL-23, a member of the IL-6 superfamily of cytokines (Oppmann et al. 2000) and data showing IL-23-deficient mice to be resistant to experimental autoimmune encephalomyelitis (EAE) (Cua et al. 2003) and CIA (Murphy et al. 2003) supported this. Naïve T cells, however, do not express high levels of the IL-23 receptor (IL-23R) (Parham et al. 2002) and it

has since been shown that whilst IL-23 is necessary for the maintenance and pathogenicity of Th17 cells (McGeachy et al. 2007), IL-23 does not actually induce Th17 differentiation.

Three studies published in 2006 showed that Th17 cell differentiation is not induced by IL-23 but instead by Transforming growth factor-β (TGF-β) in concert with IL-6 (Bettelli et al. 2006;Veldhoen et al. 2006;Mangan et al. 2006). IL-23 was instead found to be necessary for Th17 cell survival and expansion. The role of TGF-β in inducing Th17 cells, so important in autoimmunity, contrasts with its role in the delineation of Treg cells when present without IL-6 (Hori, Nomura, & Sakaguchi 2003;Chen et al. 2003).

Secreted by Treg cells and usually regarded as a key immunosuppressive cytokine, TGF-β has an inhibitory effect on effector T cell proliferation (Li et al. 2006) but, conversely, induces production of auto-immunogenic Th17 cells when present together with IL-6. TGF-β can be an inducer of immunosuppressive Treg cells or immunogenic Th17 cells depending on IL-6 concentration. Hence, either cell type, but not both are produced. The implications of this so-called reciprocal relationship between Th17 cells and Treg cells (Bettelli et al. 2006) will be considered below.

1.1.3 *Peripheral tolerance*

Effector CD4+ T cells of each subtype initiate an adaptive immune response after they become activated, which causes clonal expansion, and enables the activated T cells to access sites of inflammation (Sallusto et al. 1999). Classically, the Two Signal model has been used to describe the conditions required to achieve T cell activation (Mueller, Jenkins, & Schwartz 1989); ligation of TCR with cognate antigen presented on MHC molecules, and costimulation provided by either surface molecules such as CD40 and CD80/86, expressed on mature (activated) APCs, or pro-inflammatory cytokines such as interleukin-2 (IL-2), or both.

Autoreactive T lymphocytes that have escaped the mechanisms of central tolerance may encounter their specific antigen in the periphery, but if this occurs in the absence of other inflammatory signals, the T cell is unlikely to initiate a damaging autoimmune reaction; if TCR ligation occurs in the absence of costimulatory signals the T cell cannot become strongly activated (Bour-Jordan & Blueston 2002;Zhou et al. 2002), and should induce no response. Although the non-inflammatory context of TCR ligation in the absence of costimulation prevents the T cell from mounting an autoimmune response, the two-signal model for T cell activation can present a problem in the ability to distinguish dangerous foreign antigens from innocuous or self antigens when a self peptide is presented in the presence of pro-inflammatory cytokines that had been produced by other activated immune cells (the bystander effect), or by an antigen presenting cell that has become activated by another stimulus.

Immature dendritic cells continually survey the extracellular environment, capturing innocuous material from dead cells (Steinman & Nussenzweig 2002). Subsequent encounter with pathogenic microorganisms at a site of infection will activate DCs through interaction with pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) (Medzhitov 2001;van Vliet et al. 2007) inducing maturation which leads to increased processing and presentation of peptides derived from both the pathogen and the innocuous material, as well as upregulation of costimulatory signals. Any T cell that had escaped deletion in the thymus and is reactive against an epitope from an innocuous antigen captured by the DC before maturation has the potential to be activated by the mature DC. However, potentially dangerous autoimmune reactions induced by autoreactive peripheral CD4+ T cells are thought to be avoided in three main ways.

After activation by a mature DC that is presenting self antigen, if the autoreactive T cell subsequently encounters an immature or non-activated APC presenting the same epitope in the absence of costimulatory signals, it can be deleted through activation induced cell death (AICD) (Kabelitz, Pohl, & Pechhold

1993;Hildeman et al. 2002;Krammer, Arnold, & Lavrik 2007) although it was recently shown that Th17 cells are largely resistant to AICD (Yu et al. 2009).

T cell anergy can be defined as a state of long-lasting, partial or total unresponsiveness induced by partial activation (Lechler et al. 2001) that is maintained *in vivo* by continuous antigen exposure (Pape et al. 1998). When a TCR is ligated but only low levels of costimulatory molecules are present on the APC, a naïve T cell will be anergised, and unable to express IL-2 or proliferate (Quill & Schwartz 1987;Schwartz 2003) even if it subsequently encounters a fully mature APC presenting its cognate antigen along with costimulatory signals. It has been shown *in vivo* that when cells in which the pro-apoptosis Bim protein - essential for AICD - has been eliminated, are continually exposed to self antigen, anergisation results in those cells that would otherwise have been deleted by AICD (Barron et al. 2008). In this study anergising self-reactive peripheral T cells was enough to maintain tolerence.

1.1.4 *Treg cells*

The third mechanism by which autoreactive T cells are prevented from inducing an autoimmune response, even when presented with their specific antigen in the presence of strong costimulation, is through antigen-specific suppression by CD4+ regulatory T cells. Protection from autoimmunity can be conferred to animals deficient in these cells by transfer of Tregs from healthy, syngeneic animals (Sakaguchi et al. 1995). Prevention of antigen-specific autoimmunity by Treg cells is an active, dynamic and multi-pronged process (Sojka, Huang, & Fowell 2008).

Treg cells inhibit proliferation and IL-2 secretion of effector CD4+/CD25- T cells (Thornton & Shevach 1998). Northern blot analysis revealed IL-2 mRNA levels in the responder CD4+/CD25- T cells was undetectable after co-culture with Tregs. Addition of exogenous IL-2 or other costimulation in the form of anti-CD28 rescued proliferation in responder T cells. Whilst this suppression appears to be contact-dependent (Thornton & Shevach 1998;Takahashi et al. 1998), IL-2

deprivation may contribute. Treg cells have been shown to absorb IL-2 via CD25, their high-affinity IL-2 receptor α-chain (de la Rosa et al. 2004;Barthlott et al. 2005). Apoptosis of effector T cells induced by cytokine deprivation has been demonstrated (Pandiyan et al. 2007).

Although IL-2 downregulation is often used as a primary readout of target cell suppression by Tregs (Sojka, Huang, & Fowell 2008), comparison of gene expression profiles between Treg-suppressed target cells and other non-proliferating T cells showed expression of many genes associated with growth arrest or inhibition of proliferation (Sukiennicki & Fowell 2006). Destabilisation of the interaction between responder T cell and APC by the Treg may play a significant role in immunosupression (Tadokoro et al. 2006). A recent report has shown that platelet glycoprotein-specific Tregs can mediate their suppressive activity by modulating DCs pre-pulsed with platelet glycoprotein. These modulated DCs displayed reduced levels of CD86 and MHC class II and were unable to stimulate platelet glycoprotein-specific responder T cells (Zhang et al. 2009).

Another contact-dependent mechanisms is one of transfer of cAMP from Tregs directly into responder T cells via gap junctions resulting in strongly reduced proliferation and inhibition of expression of key cytokines IL-2 and IFN-γ (Bopp et al. 2007).

Despite the initial focus on contact-dependent Treg action (Thornton & Shevach 1998;Takahashi et al. 1998) and *in vitro* studies showing that elimination of IL-10 and TGF-β did not abrogate Treg function (Dieckmann et al. 2001;Jonuleit et al. 2001), these immunosuppressive cytokines, secreted by many Tregs, have been shown to be crucial for Treg function in many *in vivo* systems (Powrie et al. 1996;Asseman et al. 1999;Hara et al. 2001;Kingsley et al. 2002;Strauss et al. 2007). IL-35, identified in 2007, found to be secreted constitutively by Tregs, but not other CD4+ T cells, was required for maximal suppressive activity (Collison et al. 2007). IL-35 production by Tregs is increased by contact with effector T cells, suggesting

that cell contact may be essential for the initiation but not maintenance of suppression (Collison et al. 2009).

1.1.5 *Generation of Tregs*

When Tregs are induced in the thymus, a strong affinity for the MHCII-peptide complex is required to select thymocytes to develop down this lineage (Jordan et al. 2001) to become what are now called natural Tregs (nTregs). In the periphery, naïve CD4+/CD25- T cells can be converted to CD25+/FoxP3 induced Tregs (iTregs) by weaker MHCII presentation in the presence of TGF-β (Kretschmer et al. 2005b) along with costimulation from CD80/86 (Liang et al. 2005) and CTLA-4 (Zheng et al. 2006). TGF-β has been shown to be the key cytokine in generating iTregs from CD4+/CD25- T cells *in vitro* when present at the time of TCR ligation (Chen et al. 2003;Fantini et al. 2004). TGF-β, however, far from being a solely immunosuppressive cytokine is also crucial for the development of Th17 cells that are important in both human RA (Shahrara et al. 2008) and CIA in mice (Nakae et al. 2003). Naïve CD4+ T cells will differentiate into Th17 cells in the presence of TGF-β in concert with IL-6 (Bettelli et al. 2006) through expression of the transcription factor RORγt (Ivanov et al. 2006). TGF-β also upregulates the IL-23 receptor conferring responsiveness to IL-23 (Mangan et al. 2006) thus assisting in the survival of Th17 cells. This iTreg/Th17 paradigm presents an interesting immunological pivot upon which damaging autoimmune reactions could be triggered or avoided. The cytokine environment, in particular the presence or absence of IL-6 at the time of TCR ligation could make the difference between differentiation of an autoreactive naïve CD4+ T cell into an autoimmunogenic Th17 cell or an immunosuppressive iTreg. By blocking IL-6 signaling in mice using anti-IL-6R mAb, inflammatory IL-17 responses are inhibited and arthritis is suppressed (Fujimoto et al. 2008). The humanised mAb against the IL-6 receptor, tocilizumab, as produced by Roche under the name RoACTEMRA, can be used as an effective adjunct therapy for rheumatoid arthritis in combination with methotrexate.

1.1.6 *Methotrexate in the treatment of RA*

First synthesised in the 1940s and described as an analogue of Pteroylglutamic (folic) acid (Seeger et al. 1949), methotrexate inhibits the enzyme dihydrofolate reductase (DHFR) that is essential in tetrahydrofolate synthesis. Tetrahydrofolate is a single carbon donor in many biosynthetic pathways including those that ultimately produce purines and pyrimidines for DNA and RNA molecules and is therefore necessary for cell proliferation. Methotrexate's inhibitory action on DHFR reduces tetrahydrofolate synthesis ultimately limiting the cell's ability to synthesise DNA, thus preventing it from undergoing mitosis. Extensively used in treating neoplastic diseases, the rational for using methotrexate for the treatment of RA was the inhibition of proliferation of the lymphocytes and other inflammatory cells in the affected joints (Cutolo et al. 2001).

In the years after several trials found methotrexate to be an effective treatment for RA (Thompson et al. 1984;Weinblatt et al. 1985) several lines of evidance suggesting that methotrexate does not simply act as an anti-proliferative started to emerge. Indeed, methotrexate had been shown to act when administered in pulses (Andersen et al. 1985). It was difficult to imagine how a therapeutic effect might be achieved by anti-proliferative levels of methotrexate being present for only a short time each week.

Anti-inflammatory mechanisms of action have been suggested (Kremer 1994) and recent discussions of the mechanisms of methotrexate's therapeutic action include triggering the release of endogenous adenosine (that itself has anti-inflammatory effects) and suppressing production of pro-inflammatory cytokines IL-4, IL-6, IL-13, TNF-α and IFN-γ (Gerards et al. 2003;Wessels, Huizinga, & Guchelaar 2008).

Other mechanisms by which methotrexate exerts an anti-proliferative effect, particularly on lymphocytes, have also been proposed; alteration of intracellular levels of reactive oxygen species and increased sensitivity of activated T cells to

CD95 have been shown to lead to apoptosis of the autoreactive T cells (Strauss, Osen, & Debatin 2002;Wessels, Huizinga, & Guchelaar 2008).

1.2 *Collagen Induced Arthritis*

CIA is an autoimmune condition that can be induced in mice, rats and monkeys. It is a well-characterised model of autoimmunity in general but also shares many key features with human Rheumatoid Arthritis.

1.2.1 *Disease induction*

It was first shown in 1977 that rats injected intradermally with collagen II (CII) derived from human, chick, or rat cartilage (that contain identical amino acid (aa) residues 256-272) in either complete Freund's adjuvant (CFA), that contains inactivated and dried mycobacteria emulsified in mineral oil, or in incomplete Freund's adjuvant (IFA), composed of the same oil but without the mycobacterial component, caused about 40% of rats to develop a chronic inflammatory arthritis (Trentham, Townes, & Kang 1977). It was also noted that injection of either CII without adjuvant, collagen type I or type III with adjuvant, or adjuvant alone was unable to induce arthritis in any rat. This is not surprising given that the only collagen present in cartilage is CII, itself forming the major constituent of articular cartilage (Brand, Kang, & Rosloniec 2004). Encoded by the single gene COL2A1, located on the long arm of chromosome 12, CII is found only in cartilage, in the vitreous humor of the eye and in the inner ear.

When collagen induced arthritis in mice was first described (Courtenay et al. 1980), the disease was again shown to be induced by CII, (in this case of bovine origin) but not collagen I. Arthritis induction in the DBA/1 mice used in this study required a second, booster injection of CII emulsified in acetic acid administered intraperitoneally (*ip*) on day 21. All male mice initially administered with CII in CFA developed clinical arthritis in one or more joints by 14 days after the booster administration. About 90% of female mice develop clinical disease by 14 days, and all by 28 days. Of mice initially administered with CII emulsified with IFA, 70% of males and just 40% of females developed clinical arthritis in one or more joint at 28

days post booster injection, and just 80% of males and 50% of females when the experiment was terminated 62 days after initial immunisation.

Autologous CII in CFA induces an arthritic condition in male but not female DBA/1 mice which more closely resembles human rheumatoid arthritis, with a more gradual onset, and periods of remission and relapse (Holmdahl et al. 1985a); (Holmdahl et al. 1986) however, a generally later but widely varying time of onset of clinical disease, along with large variability in histological data and key markers such as anti-CII titre between individual mice (Holmdahl et al. 1986) limits the usefulness of using mouse CII-induced arthritis as a robust and repeatable model of autoimmunity.

The present study has induced arthritis in male DBA/1 mice by intradermal administration of 100µg rat CII emmulsified in CFA followed 21 days later by an *ip* booster injection of 100µg rat CII in IFA.

1.2.2 *Antibody response*

In 1981 Wooley *et. al* observed high titres of anti-CII antibodies not only in B10-Q and DBA/1 mice that developed arthritis, but also in other strains of mice that had received exactly the same immunisation, but that never developed clinical signs of inflammation (Wooley et al. 1981) suggesting that the presence of anti-collagen antibodies is not itself sufficient to cause disease. Only mouse strains bearing the MHCII I-A^q haplotype were susceptible to CIA. The Th1-associated IgG2a isotype was the only antibody to show raised serum levels in arthritic mice but not non-arthritic mice, and these antibodies are essential for the establishment of joint inflammation which is mediated by their ability to fix complement (Watson & Townes 1985).

Whilst transfer of sera or purified anti-CII Abs from mice that have developed CIA to recipient mice rapidly initiates severe arthritis (Holmdahl et al. 1990), an effect termed α-collagen antibody induced arthritis (CAIA), this effect is not dependent on the host MHCII I-A haplotype but on the presence of a functional

complement pathway (Nandakumar & Holmdahl 2006) such that complement protein C3 can be activated (Banda et al. 2007). This demonstrates that binding of arthritogenic immunoglobulins to collagen contributes to arthritis development. However, the necessity for the donated immunoglobulins to be of the complement-fixing subtypes, and the restriction to mice of particular I-A haplotypes of the ability to produce large amounts of these antibodies in response to exogenous CII implies that initiation of arthritis is dependent on properties of the immune system upstream of B cell maturation and antibody production. It is clear that the antibody response is a necessary part of the pathogenesis of CIA as B cell-deficient mice do not develop CIA (Svensson et al. 1998).

Arthritogenic mouse monoclonal anti-collagen antibodies recognise surface-exposed epitopes present on native, triple helical, but not denatured collagen that share a common "proline-glycine-hydrophobic" amino acid motif (Schulte et al. 1998). Human IgG in sera from RA patients have been found to recognise to the same epitopes as arithritogenic mouse mAbs from CIA (Burkhardt et al. 2002) and antibodies purified from RA patients can cause a transient arthritis when injected into mice (Wooley et al. 1984). As discussed in **1.2.6**, CII is heavily post-transcriptionally modified. This actually increases recognition by IgG in sera from RA patients compared to binding to unmodified CII.

1.2.3 *Clinical and histological manifestations*

Murine CIA is characterized by an intense synovitis that corresponds with the clinical onset of arthritis (Brand, Kang, & Rosloniec 2004). Following an early phase in which T cells and MHC class II-expressing macrophages infiltrate the synovial lining, oedema accompanies infiltration of large numbers of granulocytes before formation of a pannus that marks the beginning of cartilage and bone destruction (Holmdahl, Tarkowski, & Jonsson 1991). Immunohistopathologic analysis of arthritic joints from CIA display important similarities with rheumatoid joints, with the formation of synovial pannus tissue being the prototypical

morphologic feature (Trentham 1982). Fibrin deposition occurs, and there is often symmetrical joint involvement persistently affecting peripheral joints causing synovial hyperplasia (Joe & Wilder 1999). As in RA, the pannus, which contributes to cartilage and bone erosion, includes many macrophages, activated dendritic cells and granulocytes (Holmdahl et al. 1988;Holmdahl, Tarkowski, & Jonsson 1991), a small number of CII-specific CD4+ T cells (Arai et al. 1996;Latham et al. 2005) as well as osteoclasts (Gravallese 2002) which are the only bone resorbing cell type and are involved in bone erosion when activated.

1.2.4 *The restriction of CIA susceptibility to mice possessing certain MHC class II alleles suggests a critical role for CD4+ T cells in disease initiation*

It was noted in the 1981 Wooley paper that the only mouse strains capable of developing clinical CIA bear the MHCII I-A^q haplotype (Wooley et al. 1981). The same group later showed that such mice were susceptible to chick, bovine, deer, rat and human type II collagen, but were largely resistant to arthritis induced by porcine type II collagen whilst mice that express the MHCII I-A^r allele develop CIA in response to bovine, deer, and porcine collagen (Wooley et al. 1985) Arthritis induction using autologous (mouse) CII results in a milder disease, described in **1.2.2**. It was postulated that the I-A^q class II molecule might bind and present one arthritogentic epitope present on chick, deer, rat and human CII whilst the I-A^r molecule might present a different arthritogenic epitope present on porcine CII and that both epitopes may be present on bovine and deer CII. Further work elucidating these immunodominant epitopes will be described below.

The I-A^p molecule only differs from I-A^q by four amino acids, the substitution of which then confers susceptibility to CIA on H-2^p mice that are otherwise refractory (Brunsberg et al. 1994). This provides direct evidence for the critical nature of MHCII-antigen interactions for disease induction.

Peptides presented in the context of MHCII molecules are bound by TCRs with an affinity for the peptide-MHCII complex such that the T cell may be primed

to respond to subsequent TCR ligation in an immunostimulatory fashion. Given the role of CD4+ T cells in the initiation of adaptive immune responses and in antibody switching, this early data strongly suggested that for a mouse to be susceptible to disease induction, the ability to present CII-derived epitopes to activate CD4+ T cells is critical.

Adoptive transfer of expanded CII-specific helper T cells can induce arthritis in syngeneic recipients (Holmdahl et al. 1985b;Kakimoto et al. 1988). The crucial role of CD4+ T cells is further supported by observations showing removal of CD4+ cells renders mice resistant to CIA. Mice administered with rat anti-mouse-CD4 immunoglobulins the day before CII immunisation, which results in depletion of 90% of CD4+ T cells, prevented 8 out of 10 mice from developing arthritis (Ranges, Sriram, & Cooper 1985). Anti-T cell receptor antibodies were subsequently shown to be an effective treatment after arthritis induction in both rats (Yoshino, Cleland, & Mayrhofer 1991) and mice (Chiocchia, Boissier, & Fournier 1991). Knockout mice for the *tcrb* locus that encodes the TCR β-chain lack any CD4+ cells at all and are completely resistant to CIA (Corthay et al. 1999). CD4 -/- mice are not completely refractory to CIA however (Tada et al. 1996;Ehinger et al. 2001) although this could be due to an abnormally high number of CD4-CD8-TCR+ cells, many found in the joints of these mice after immunological challenge with CII, that may contribute to disease (Tada et al. 1996).

1.2.5 *A CII-derived peptide, recognised by the MHCII-I-A^q allele, was shown to be an immunodominant epitope, and renders mice resistant to CIA when exposed to it during early development*

Building on work that showed that the only cyanogen bromide digestion fragment from chick CII able to induce arthritis in DBA/1 mice was $CII_{245\text{-}270}$ (Terato et al. 1985), Myers' group in the early 1990s used amino acid substitutions on synthetic peptides to show that an immunodominant 11-mer $CII_{260\text{-}270}$ drives a Th1-like response when incubated with splenocytes cultured from DBA/1 mice pre-

immunised with chick CII (Myers et al. 1992). They further demonstrated that administering the same peptide to neonatal mice induces collagen-tolerance, making them refractory to CIA. This peptide is not recognised by mouse strains that do not express the I-A^q allele (Gustafsson et al. 1990;Myers et al. 1993). Rat, bovine, human and chick are identical at positions 256-273 (figure 1.2).

	256			259					264		266				270			
Rat:	G	E	P	G	I	A	G	F	K	G	E	Q	G	P	K	G	E	T
Bov:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P
Mus:	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-
Hum:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P
Chic:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pig:	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P

Figure 1.2 Sequence alignment of amino acid residues 256-273 of CII. Comparison of 6 species – rat, cow, mouse, human, chicken and pig.

Further evidence supporting the immunodominance of this collagen determinant is provided by experiments showing that rat CII is unable to induce arthritis in transgenic mice that express the $CII_{256-270}$ fragment in their Collagen type I (CI), denoted TSC mice.

Draining lymph node cells from TSC mice are unable to respond to rat CII whereas draining lymph node cells from MMC-1 mice that have point mutation D266E, the one amino acid residue in the $CII_{256-270}$ epitope that differs between mice and human, rat, bovine and chick CII, do produce IFN-γ and normal anti-CII immunoglobulin levels but do not proliferate in response to rat CII suggesting that the $CII_{256-270}$-reactive T cells were not eliminated, but tolerised (Malmstrom et al. 1996). This difference in the magnitude of tolerisation induced by either expression

of the heterologous $CII_{256-270}$ epitope ectopically in the systemically occurring CI molecule or in place of the autologous CII epitope in MMC-1 mice (that still develop severe arthritis in response to rat CII, albeit with a halved rate of incidence) is thought to be due to only peripheral part-tolerisation that can be achieved by expressing the heterologous epitope in CII which is resident only in cartilage and the vitreous humour of the eye. It was later shown that chick CII can induce arthritis in TSC mice dependent on the subdominant, I-A^q-restricted chick-$CII_{190-200}$ epitope (Malmstrom, Kjellen, & Holmdahl 1998).

The arthritogenic response of I-A^r-bearing mice focuses on another collagen epitope, $CII_{442-455}$, but the susceptibility of I-A^q mice is heavily focussed on TCR recognition of the $CII_{256-270}$ epitope bound in the peptide-binding groove of Class II I- A^q molecules (Rosloniec et al. 1996;Kjellen et al. 1998).

The $CII_{256-271}$ epitope is likely important in RA. Peripheral blood mononuclear cells (PBMCs) of RA patients react to $CII_{256-271}$ and its overlapping variants (Ohnishi et al. 2003).

1.2.6 *Posttranslational modifications of CII by enzymes such as LH3 alter the immunogenicity of the immunodominant epitope*

Lysine residues at the Yaa position of the Gly-Xaa-Yaa triplet of collagen molecules are hydroxylated at varying frequencies, converting Gly-Xaa-Lys aa sequences to Gly-Xaa-Hyl, a process essential for inter-molecular cross-linking that endows fibrils with strength (Reiser, McCormick, & Rucker 1992). Some hydroxylysine (IUPAC name 2,6-Diamino-5-hydroxyhexanoic acid, formula $C_6H_{14}N_2O_3$) residues are then further modified by addition of carbohydrates (glycosylation).

The structure of the major glycan on collagen was solved in 1967 as being α-glucosyl-1,2-β-galactopyranose (Glc(α1-2)Gal(β1-O)) (Spiro 1967). Lysyl-hydroxylase 3 (LH3) can perform both the initial hydroxylation (Valtavaara et al. 1998) and has been reported to further modify collagen via a glycosyl-transferase

domain distinct from the hydroxylating site, conjugating a β-galactopyranose monosaccharide (Gal(β1-O)) or a Glc(α1-2)Gal(β1-O) disaccharide to the hydroxylated lysine residue (Heikkinen et al. 2000). Such modifications of the Lys264 residue of the immunodominant peptide of CII ($CII_{259-271}$) have important implications for the recognition by CD4+ T cells of the MHCII-bound peptide. Although recent reports suggest the glycosyl-transferase activity of LH3 is modest (Schegg et al. 2009), the present study has shown that coexpression of LH3 with fusion proteins that include the CII peptide domain can enhance glycosylation of the $CII_{259-271}$ peptide.

Four post-transcriptional modification states of the Lys264 residue exist, namely the unmodified (naked) lysine residue, hyroxylysine, Gal(β1-O)-hydroxylysine, and Glc(α1-2)Gal(β1-O)-hydroxylysine. When bound in the Class II pocket, these Lys264 variants result in at least four different T cell-determinants, the Gal(β1-O)-hydroxylysine version being recognised by the majority of T cell clones from immunised mice (Corthay, Backlund, & Holmdahl 2001). Analysis of clonally expanded (and hybridised) T cells from two strains of $I\text{-}A^{q}$+ mice strains found 20/29 specifically recognized CII(256–270) glycosylated with the monosaccharide Gal(β1-O) suggesting that this glycopeptide is immunodominant in CIA (Corthay et al. 1998). Removing the sugar moiety by biochemical methods disrupts recognition of the MHC-peptide complex by certain T cell hybridomas, showing the importance of glycosylation in inducing a response from these T cell lines (Michaelsson et al. 1994).

Incidence and mean arthritic score in $(B10.QxDBA/1)F_1$ male mice immunised with biochemically de-glycosylated rat CII is reduced as compared to untreated rat CII, and time of onset is also delayed (Michaelsson et al. 1994). A similar dependence on glycosylation state of the tolerising potency of the $CII_{259-273}$ peptide has been demonstrated when using it bound within the peptide-binding groove of soluble $I\text{-}A^{q}$ molecules. Such peptide-MHC complexes have preventative

and therapeutic effects in CIA model only when the $CII_{259-273}$ peptide is glycosylated in the 264 position (Dzhambazov et al. 2006).

It has been suggested that where PTAs are post-transcriptionally modified in the periphery, they may not be presented by MECs in the thymus in all isoforms, and the glycosylation of collagens may be one scenario (Malmstrom, Trollmo, & Klareskog 2004) where the negative selection of autoreactive T cells may not extend to those T cells that recognise the epitope in all its post-translationally modified isoforms.

1.3 *Clinical need for tolerance induction*

Autoimmunity, graft rejection and graft-versus-host disease are three major clinical conditions that are currently treated with immunosuppressives as a first-line measure. Whilst this can be effective in controlling symptoms, it can leave the patient vulnerable to infection and does not address the underlying cause of the undesirable immune reaction. Hence, induction or restoration of tolerance are major therapeutic goals.

1.3.1 *Experimental induction of antigen-specific tolerance*

The first report of experimentally generated tolerance by Medawar in 1953 showed that *ip* injection of allogeneic cells into fetal mice facilitates acceptance in adulthood of skin grafts from mice of the cell-donor strain (Billingham, Brent, & Medawar 1953). Similar results were obtained in chickens and the induction of tolerance in this way was shown to be dependent on the exposure to foreign antigens whilst the host's immune system is still in an immature state (ie before birth). Induction of specific tolerance to allogeneic bone marrow graft in adult mice was described by Slavin *et al* in 1977 by total lymphoid irradiation of recipient mice prior to infusion of bone marrow cells from donor mice resulting in specific tolerance to skin grafts of donor-strain origin (Slavin et al. 1977). A year later, the same group showed that tolerance to BSA following total lymphoid irradiation and subsequent *ip* BSA administration was mediated by antigen-specific suppressor cells (Zan-Bar, Slavin, & Strober 1978). Immunosuppressive T lymphocytes were finally characterised in 1995 as coexpressing CD4+ and CD25+ and were called Treg cells (Sakaguchi et al. 1995).

Many experimental strategies to develop protocols for tolerance induction have focussed on Treg induction and expansion. There are multiple subclasses of Treg cells, some of which can be generated or expanded in an antigen-specific manner.

Treg cells can be induced both in the thymus (Jordan et al. 2001;Picca & Caton 2005) and in the periphery (von Herrath & Harrison 2003;Kretschmer et al. 2005b) and there have been many reports of conversion of CD4+/CD25- T cells into CD4+/CD25+/FoxP3+ Tregs *in vitro* (Zheng et al. 2002;Zheng et al. 2004;Zheng et al. 2006).

Conversion of CD4+/CD25- T cells into Tregs can be achieved by targeting minute doses of cognate peptide to DCs for suboptimal activation, thus generating large numbers of antigen-specific immunosuppressive cells (Mahnke et al. 2003). Over 50% of CD4+/CD25- T cells can be converted to Tregs (Bettelli et al. 2006).

Antigen-specific regulatory T cells that do not express FoxP3, termed Tr1 cells can be induced by culturing murine or human $CD4^+$ T cells with antigen or alloantigen in the presence of exogenous IL-10 (O'Garra & Vieira 2004;Vieira et al. 2004). CD25-negative Th3 cells are also experimentally induced in an antigen-specific manner via TCR ligation during transient overexpression of TGF-β (Carrier et al. 2007), but mediate suppression by secreting TGF-β and so are suppressive in an antigen-independent manner, thereby potentially giving rise to "bystander suppression"(Weiner 2001). Nonetheless, this, along with each of the other methods for generating antigen-specific Treg cells, have potential uses in establishing antigen-specific tolerance in transplantation and autoimmune diseases including autoimmune arthritis (Thomson & Robbins 2008).

A method of expanding nTregs in the periphery of live mice has recently been reported (Swee et al. 2009). Achieved by injection of supra-physiologic injection of Fms-like tyrosine kinase 3 ligand, a cytokine required for DC homeostasis that results in massive DC expansion when present at these levels, this indirectly induces a two-fold increase in nTregs as a result of the 18-fold expansion of DCs. Whilst the expanded nTregs were demonstrably one fraction of previously existing nTregs in the periphery that had been induced to proliferate, there is no selectable antigen specificity of these cells.

Whilst FoxP3+ Treg cells have been generated *in vitro* by using TGF-β in concert with TCR costimulation (Chen et al. 2003), antigen presentation by immature dendritic cells in the "steady state" is increasingly implicated in Treg generation (Yamazaki et al. 2003;Gaudreau et al. 2007). Even experimentally-matured DC's can stimulate Treg generation in an antigen-specific fasion (Gilliet & Liu 2002;Lau et al. 2008).

Antigen-specific tolerogenic DCs, prepared by pulsing immature DCs with influenza matrix peptide (MP) has been used to induce MP-specific tolerance in human test subjects when injected subcutaneously (Dhodapkar et al. 2001). This was found to induce suppressor CD8+ T cells, and the silencing of CD8+ effector cells was found to be specific for MP-reactive T cells. Antigen-specific Tregs that may have important uses in transplant patients have been generated by culture with allogeneic DCs in the presence of IL-10 (Levings et al. 2005).

Such methods may represent a viable approach for the conversion of antigen specific conventional T cells into Treg cells *in situ* in the periphery in a similar way as *de novo* generation in the periphery via conversion of truly naïve CD4+ helper cells has been achieved by targeting small amounts of specific antigens to be presented by immature DCs (Mahnke et al. 2003;Kretschmer et al. 2005a;Jaeckel et al. 2006). In this way, it is hoped, by expanding antigen-specific regulatory T cells, antigen-specific tolerance could be generated.

An alternative method for antigen-specific tolerance induction, rather than trying to generate Tregs directly, or by using tolerogenic DCs has been to perform systemic administration of soluble antigen. This has been done by single or multiple *iv* or *ip* injections in the absence of adjuvant. This has been shown to be effective in both Experimental autoimmune encephalomyelitis (EAE) (Levine et al. 1972) and type 1 diabetes (Tisch, Wang, & Serreze 1999;Atkinson & Leiter 1999). Mechanisms include anergy (Gaur et al. 1992) due to TCR ligation in the absence of costimulation usually provided by adjuvant (Boussiotis et al. 1993) and Treg cell induction (Thorstenson & Khoruts 2001). Beneficial effects in clinical investigations

of antigen therapy for allergy and chronic progressive multiple sclerosis at the end of the 1990s, however, were disappointing (Warren, Catz, & Wucherpfennig 1997;Norman et al. 1997;Bousquet et al. 1998).

The oral route of antigen exposure can also be effective in preventing the induction of autoimmune diseases (Khoury et al. 1990;Zhang et al. 1991). In 2007 the $CII_{250-270}$ peptide, administered via the oral route, was shown to be immunosuppressive in CIA (Zhu et al. 2007). Several mechanisms that mediate this effect have been suggested including deletion and anergisation of CD4+ cells and Treg induction (Mowat et al. 2004). No significant beneficial effects were observed in phase II clinical trials that attempted to induce oral tolerance using bovine or chicken CII in RA patients however (Trentham et al. 1993;McKown et al. 1999;Staines et al. 2004) although oral insulin administration did delay the onset of diabetes in high-risk patient populations (Chaillous et al. 2000).

Altered peptide ligands (APL) are peptides that vary from immunodominant epitope peptides by one or two amino acids. Such peptides have been shown to act "antagonistically" to T cell clones (Sloan-Lancaster & Allen 1996) and administration of such peptides has been shown to be effective inhibitors of EAE in mice (Franco et al. 1994). Whilst administration of APL at the time of EAE induction profoundly suppressed disease, because the immune response in humans is polyclonal, a ligand that acts as an antagonist to one T cell clone may stimulate another. A phase II clinical trial for the use of myelin basic protein APL was halted when disease was exacerbated in some MS patients (Bielekova et al. 2000).

1.3.2 *Gene therapy approaches for antigen-specific tolerance induction*

Gene therapy has been used in imaginative and diverse ways to drive antigen-specific tolerance in experimental animals. Loosely defined as the transfer of genetic material into cells for therapeutic gain (see **1.5.5** *Gene therapy in humans*, below), a slightly narrower definition is *the insertion of a gene into a cell* – a definition that excludes oligonucleotide delivery such as some RNAi technologies.

Whilst two oligonucleotide therapies undergoing human clinical trials will be mentioned briefly in section **1.5.7** *Gene therapy for rheumatoid arthritis* below, this overview of gene therapy approaches for inducing antigen-specific tolerance in experimental animals will only consider studies that insert recombinant genes.

The simplest form of gene therapy involves transfer of plasmid DNA into cells. Typically injected intramuscularly, often into the quadriceps, naked plasmids are cytophagocytized, and their encoded genes expressed (Wolff et al. 1992). Expressed genes can be presented on MHC class I by myocytes or on MHCII by neighboring APCs. Because such presentation occurs without inflammation, the vaccine can be tolerising due to the lack of costimulatory molecules. DNA vaccines encoding a peptide fraction of myelin proteolipid protein have been shown to be protective against EAE (Ruiz et al. 1999), and plasmids expressing mouse CII (Ho et al. 2006) or chick CII (Song et al. 2009) were protective against CIA in mice and rats respectively.

Of critical importance to the design of this project was a publication from 2006 on transfecting DCs with DNA vaccines (Maksimow et al. 2006). By fusing the endosomal-trafficking motif from LAMP-1 onto the OVA model antigen, DCs expressing this fusion construct did not prime an immune response against OVA-expressing lymphoma cells in recipient mice but rather attenuated the response – tolerance to OVA was actually enhanced. This was shown to be Treg cell-mediated. Also included on the fusion construct was an eGFP domain, immediately proceeding the LAMP-1 signal sequence at the 3'-end, that enabled the authors to identify transfected DCs and optimize their transfection protocol. The authors conclude that by driving OVA antigen presentation in the context of MHCII, tolerance to OVA was induced.

The use of viruses in gene therapy allows efficient and sometimes cell-specific DNA transfer. Of particular interest to this introduction are the family *retroviridae* that includes lentiviruses. The genome of retroviruses is single-stranded RNA that is reverse-transcribed within the cell and the resultant DNA is integrated

into the host genome. This has the advantage of guaranteeing permanent presence of the transgene both within the transduced cell and in any daughter cells should it divide. HIV-1-based *lvv* are the gene-delivery vectors used in this study.

Retroviral gene therapy has been used transduce bone marrow cells with antigen-expressing vectors to induce antigen-specific tolerance for over fifteen years (Sykes et al. 1993;Ally et al. 1995). It was shown that by simply expressing a model antigen in re-infused bone marrow cells, T cells that were reactive to that antigen were deleted (Ally et al. 1995). Retroviruses have also long been used to express chimeric proteins that display either whole antigen or immunodominant epitope fused to the extracellular domain of IgG heavy chains in either bone marrow (Kang et al. 1999) or LPS-stimulated B cells (Zambidis & Scott 1996;Agarwal et al. 2000). Whilst this type of antigen presentation was shown to mediate Treg expansion (Song et al. 2004), the exact mechanism for this is unclear.

Gene and cell therapy can be used in combination to induce antigen-specific tolerance. Some such strategies rely on generating an antigen-specific cell that homes to the site of inflammation by virtue of its acquired specificity but is engineered to deliver an immunotherapeutic payload. Alternatively, APCs presenting self antigen have been engineered to deliver apoptotic signals to any effector T cell that interact with them: Collagen presenting DCs, generated *in vitro* by pulsing with CII, were transduced with adenoviruses that express TRAIL, an apoptosis-inducing ligand. In this way, CII-reactive lymphocytes that interact with the modified DCs will undergo apoptosis. Treatment of DBA/1 mice with such DCs significantly reduced CIA disease and splenic T cell response to bovine CII (Liu et al. 2003).

Recent reports have used lentiviral vectors to induce antigen-specific tolerance. *Ex vivo* transduction of DCs with *lvv* encoding the OVA model antigen under control of a novel DC-specific promoter (DC-STAMP) resulted in deletion of most OVA-reactive CD4+ T cells and also some deletion of OVA-reactive CD8+ T cells. Lenti-vectors have also been used by our group (*iv*) (Gjertsson et al. 2009) and others (*ex vivo*) (Eixarch et al. 2009) to express immunodominant peptide

autoantigens in chimeric Ii in place of the CLIP domain. This strategy ensures that the peptide epitope will be positioned in the MHCII peptide-binding groove. These studies have shown the antigen-specific tolerogenic effects of using lentiviral vectors to drive MHCII-presentation of autoantigens. The work from our group has shown that presentation of the $CII_{259-273}$ epitope on MHCII has protective effects in CIA.

The present study has also shown that by using lentiviral vectors *iv* to induce MHCII-presentation of the $CII_{259-273}$ peptide in mice, specific tolerance to the $CII_{259-273}$ is induced without the need for *ex vivo* transduction of APCs. The chimeric proteins in this study, however, have not directly inserted the $CII_{259-273}$ peptide into the MHCII peptide-binding groove by virtue of Ii-chimeras but, rather, constructed rationally designed fusion proteins that include endosomal-trafficking motifs and proteolytic cleavage sites such that the $CII_{259-273}$ peptide is released and loaded onto MHCII molecules via pathways present in APCs. Mice administered with these vectors were partially but significantly protected from clinical arthritic symptoms of CIA, namely inflamed joints.

1.3.3 *Fusion proteins used in this study, designed to induce antigen-specific tolerance*

The fusion constructs used by the present study were rationally designed, based on the Maksimow strategy (Maksimow et al. 2006), to present the $CII_{259-273}$ peptide on MHCII molecules of APCs. They are comprised of the signal peptide from LAMP-1 (which is cleaved prior to egression from the ER) fused to eGFP, the $CII_{259-273}$ epitope of interest, and finally the lysosyme-localisation transmembrane tail of LAMP-1 or HLA-DM that is at the carboxy-terminal end (figure 1.3). Also included are cathepsin S cleavage sites (Ruckrich et al. 2006) flanking the $CII_{259-273}$ epitope to facilitate efficient release for loading onto the MHCII molecule; leucine, arginine, methionine and lysine on the N-terminus side, and glutamate, asparagine, leucine and lysine on the C-terminus side. The inclusion of eGFP in these chimeric

proteins enables both the identification of cells positive for construct expression and for imaging the intracellular location of the protein product.

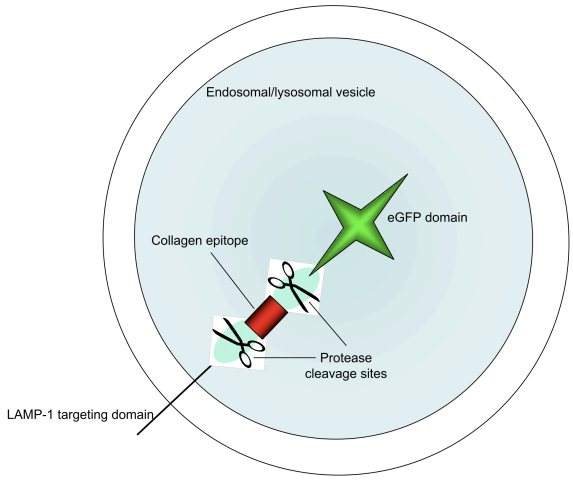


Figure 1.3 Cartoon of LAMP1-targeted fusion protein

Proteins destined for the cytosol are encoded by free ribosomes whilst those estined for secretion, for the lumen of the ER, Golgi or lysosomes, or for the membrane of any of these organelles or the plasma membrane are synthesized on the membrane bound ribosomes of the rough ER (Blobel & Dobberstein 1975). They are then targeted to the appropriate cellular compartment.

Secretory and membrane protein targeting is thought to be largely regulated during translocation through the ER. Nearly all proteins destined for any of the locations reached by the secretory pathway are targeted to the ER membrane as they are synthesized, a process called cotranslational translocation. In cotranslational translocation, signal sequences within a nascent polypeptide are recognized by universally conserved factors in the cytosol and membrane to mediate their selective targeting, translocation, and/or membrane insertion (Hegde & Kang 2008). This process is initiated when the hydrophobic core of a signal sequence or TMD emerges from the ribosome and is recognized by the signal recognition particle (SRP). The SRP-bound ribosomenascent chain complex (RNC) is then targeted to the ER

membrane via an interaction with the SRP receptor (SR). The RNC is then transferred to an adjacent translocon and the SRP – SR complex dissociates. The ER signal sequence is cleaved off from most secreted proteins by a signal peptidase on the lumenal side of the ER membrane. These steps, collectively referred to as targeting (Shan & Walter, 2005), result in the delivery of translocation substrates to sites of translocation at the ER.

Proteins synthesized on ER-bound ribosomes go to the cell surface by default, unless they are specifically directed to other compartments by special signals. Proteins that are to be retained in the ER or Golgi, or that are to be directed to specialized secretory vesicles, or to the lysosome must be sorted from those constitutively secreted (Atkinson 2006). Discrete signals on the target protein that are recognized by specific receptors actively mediate sorting of the target protein (Marks et al. 1996). Most such signals consist of short linear sequences of aa residues. Some, referred to as tyrosine-based sorting signals, are based on NPXY or YXXO (where 'O' denotes an hydrophobic residue) motifs whilst others, called dileucine-based signals fit DEXXXLLI or DXXLL consensus motifs (Bonifacino & Traub 2003).

LAMP-1 is a type-1 transmembrane protein so the signal peptide that is responsible for inverting the protein into the ER during translation is present at the amino terminus (the 5' end of the mature mRNA), whilst the intracellular trafficking of LAMP-1 to the lysosome relies upon a tyrosine-based lysosomal targeting signal GYQTI on the cytoplasmic tail mediates sorting into vesicles (Fukuda 1991; Honing et al. 1996).

The first chimeras constructed during this project used the N-terminus signal sequence from IL-2, as included upstream from the multiple cloning sites of the plasmid pFUSE-hIgG4-Fc2, Invivogen (San Diego, CA), which, being derived from a secretory protein should direct the polypeptide to the ER and enable cleavage and trafficking through the endosomal pathway. These constructs were generated as described in *Materials and Methods* below, and were shown to induce a pattern of

eGFP expression within transiently transfected cells, but the lentiviral vectors that were made for stable delivery of these constructs were unable to demonstrably transduce cells. The IL-2 signal sequence was then replaced with the signal sequence from LAMP-1 after which the lentiviral constructs, described below, were successful in stably transducing fibroblast cell lines.

The second set of constructs were generated using the endosome/lysosome-targeting moiety from mouse H2-DMβ (Potter et al. 1999), the membrane-anchored subunit of the molecular chaperone that is responsible for facilitating the loading of antigen fragments onto the MHCII molecules in the lysosomes (Vogt et al. 1999).

Lentiviral vectors were deemed to be ideal for this research project. Lentiviral transduction integrates the transgene into the host genome of even non-dividing cells, and does not alter DC maturation or function (Esslinger, Romero, & MacDonald 2002;Breckpot et al. 2003). Hence, terminally differentiated APCs will express the transgene whether they themselves are directly transduced, or if a parent precursor cell was transduced prior to division and differentiation. The biology of lentiviruses and of retroviruses in general will now be discussed, and their uses for gene therapy will be considered.

1.4 *Viruses*

Viruses are the most abundant biological entities on the planet. Most viruses infect bacteria and are called phages, outnumbering bacteria by an order of magnitude in most environments (Wommack & Colwell 2000). Viruses are genetically extremely diverse and there is no single gene common to all viral genomes (Edwards & Rohwer 2005). All viruses do, however, consist of a viral genome protected by coat proteins, and some viruses also have a lipid envelope. Many viruses are pathogenic to humans causing diseases ranging from the common cold to cancer and AIDS.

Viral genomes are comprised of DNA or RNA molecules that use the standard genetic code that ultimately translates into peptide sequence and thus, protein structure. Larger viruses such as Herpes Simplex virus can have genomes that encode over 100 proteins, whilst small RNA viruses such as the MS2 bacteriophage, that has a genome length of 3,569 bp, encodes just 4 proteins. The genome of an RNA virus can be double-stranded or single stranded which can act as a direct template for protein synthesis exactly as host mRNA does (+ssRNA virus) or require synthesis of complementary RNA, by an RNA-dependent RNA polymerase, that then acts as the template for transcription (-ssRNA virus). Retroviruses also contain an RNA genome but are often not classed as RNA viruses because the RNA genome undergoes reverse transcription to synthesise the DNA sequence that is then integrated into the host genome.

The integration processes makes retroviruses particularly useful for many types of gene therapy – if retrovirus-based vectors are used, not only is the therapeutic transgene delivered into the cell, but also integrated into the host genome. The key stages of virus life-cycle and biology will be considered below with special attention paid to HIV, on which the lentiviral vectors used in this study were based. Gammaretrovirus MLV, a simple-genome retrovirus will also be considered as gammaretroviral vectors have been widely used for gene therapy. The

advantages and disadvantages of genomic integration will also be discussed in the section on *Retroviral vectors* below.

1.4.1 *Viral entry*

Viruses enter the cell by a variety of pathways; endocytosis, membrane fusion, or, in the case of many bacteriophages, direct RNA injection. HIV enters following adsorption to and fusion with the cell membrane (figure 1.4). Adenoviruses and Adeno-associated virus (AAV) enter the cell via endocytosis that follows binding of viral coat proteins with host cell receptors and integrins (Waehler, Russell, & Curiel 2007). AAV then requires an acidic environment to escape from the early endosomes and into the cytosol (Bartlett, Wilcher, & Samulski 2000). Retroviruses enter the cytoplasm via fusion of the envelope with a cell membrane. HIV-1 surface glycoproteins interact with the CD4 molecule of T cells (Dalgleish et al. 1984) and also require interaction with chemokine receptors CXCR4 (Feng et al. 1996) or CCR5 (Dragic et al. 1996).

Pseudotyping is the exchange of viral envelope and attachment proteins from one viral strain to another (Waehler, Russell, & Curiel 2007). This can confer altered viral tropism and higher viral titres, hence, it is widely used in generating viral vectors for gene delivery. It was first shown in 1972 that VSV-G could be used to pseudotype viruses (Zavada 1972), and this is the most common retroviral and lentiviral pseudotyping envelope protein used for vector production as it results in high titre vector production and a broad tropism (Cronin, Zhang, & Reiser 2005) although VSV-G pseudotyped retroviruses do require endocytosis and acidification of endocytosed vesicles for membrane fusion to occur (Campbell et al. 2007).

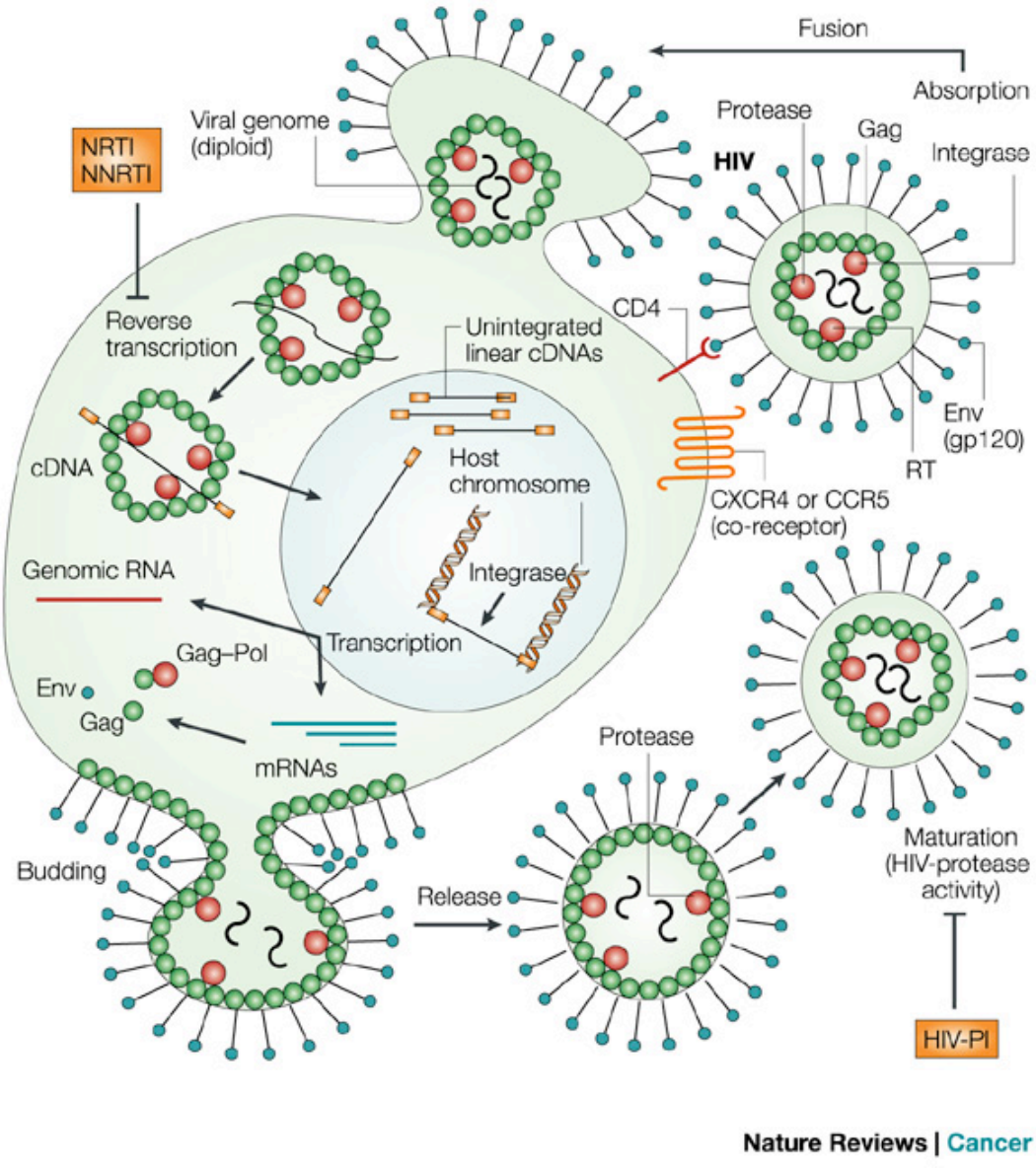


Figure 1.4 Cartoon of HIV life-cycle. HIV envelope (Env) protein gp120 binds CD4 leading to further interactions between gp120 and CCR4/CXCR5. Viral and cell membranes fuse releasing the viral core into the cytoplasm and reverse transcription is initiated, generating viral cDNA. Following transport into the nucleus, viral cDNA is inserted into the host genome by the viral protein Integrase. Transcription of viral cDNA generates genomic RNA and mRNA that is translated into the HIV envelope protein Env and immature polyproteins Gag and Gag-Pol, that, together with genomic RNA are assembled as viral progeny that bud from the cell membrane. Picture taken from www.nature.com/nrc.

1.4.2 *Uncoating and RTC formation*

Once the viral membrane has fused with a cellular membrane, the viral core (capsid) is released into the cytoplasm. For some retroviruses, the disassemblement of the viral capsid appears to be essential before reverse transcription of retroviral genome can proceed.

In HIV infection, the host protein cyclophilin A (CypA) binds to CA and it was initially thought that this interaction could be involved in either viral entry or uncoating (Luban et al. 1993). Recent studies suggest that the CypA-CA interaction is important in uncoating the viral core after entry (Yamashita & Emerman 2009;Arfi et al. 2009). The remaining complex of viral genome and viral proteins are termed the reverse transcription complex (RTC). Whilst the uncoating process is relatively rapid in HIV, in MLV the CA protein is associated with the RTC and is present throughout reverse transcription (Fassati & Goff 1999). Even in HIV-1 where uncoating is rapid, reverse transcription is thought to commence together with, not after uncoating (Arhel et al. 2007). The RTC always contains IN and RT and in HIV-1 also contains MA, NC and Vpr (Nermut & Fassati 2003). The genomic structure of retroviruses determines the process of reverse transcription and arrangement of the resulting dsDNA genome that is ultimately integrated into the host genome.

1.4.3 *Retrovirus genome structure*

Most retroviral genomes contain three main open reading frames, *gag, pol* and *env* downstream from the repeat (R) and unique 5' (U5) sequences. Almost directly adjacent to the U5 region is the primer binding site (*PBS*) from which reverse transcription is initiated. Complex retroviruses such as lentiviruses include several other reading frames that encode regulatory and accessory proteins. Downstream from the coding regions is a poly-purine tract (PPT), a 3' unique sequence (U3) and a second R sequence. The U3 sequence contains the promoter

and enhancer elements, thus, these are only upstream of the coding sequences after reverse transcription (Shinnick, Lerner, & Sutcliffe 1981).

The R sequences are important in the second step of the reverse transcription process where the first part of synthesized DNA (complementary to the 5' end of viral RNA) transfers to the 3' end of the viral RNA by complementarity with the R sequence there. The R sequences ultimately comprise the central region of the long terminal repeats (U3, R, U5) that flank the dsDNA provirus that is integrated into the host genome.

1.4.4 *Reverse transcription*

Reverse transcription is initiated by binding of host tRNA to the *PBS*. While reverse transcription proceeds in the cytoplasm of the infected cell, tRNA-*PBS* binding can occur within the virion (Trono 1992) because retroviral virions encapsulate many host tRNAs. Different host tRNA molecules are used by different retrovirus genera to initiate reverse transcription; HIV uses $tRNA^{Lys3}$ (Jiang et al. 1993) while MLV uses $tRNA^{Pro}$ (Peters et al. 1977).

The viral enzyme reverse transcriptase (*RT*), using the tRNA as a primer, begins synthesis of the minus-strand DNA complementary to the U5 and then R regions at the 5' end of the RNA genome. As well as DNA polymerase activity (Baltimore 1970), *RT* also possesses a ribonuclease-hybrid (RNase H) domain (Molling et al. 1971) that degrades template RNA after it is reverse transcribed. This initial DNA transcript is called the minus strand strong stop DNA (-sssDNA). Once its template RNA has been degraded the –sssDNA "jumps" to the 3' end of the remaining RNA genome where the R sequence of the –sssDNA pairs with the R sequence at the 3' end of the remaining RNA genome (Mitra et al. 1979).

RT, now using the –sssDNA as a primer, reverse transcribes the template RNA in the 5' direction, thus encoding the viral genes into DNA, and also degrading the template RNA with the exception of the PPT that acts as the primer for plus-strand DNA (+DNA) synthesis using the –DNA as a template (Smith, Cywinski, &

Taylor 1984). Additionally, in some retroviruses, other RNA template regions are left undegraded (such as the central poly-purine tract, cPPT, in HIV) and are used as primers to synthesise other, discontinuous +DNA strands (Charneau & Clavel 1991). Synthesis of the +DNA proceeds up until the tRNA which is then degraded by the RNase H activity, enabling a second, intramolecular jump such that the PBS at the 3' end of the –DNA strand anneals with the newly synthesized PBS on the +DNA strand allowing each strand to prime off each other. The resulting dsDNA viral genome has a Long Terminal Repeat (LTR) at each end that is blunt-ended and serves as a substrate for integration (Brown et al. 1989).

In HIV, the region of the +DNA strand synthesized from the cPPT becomes displaced by the +DNA strand primed from the PPT. The +DNA strand primed from the PPT does not completely displace the cPPT-primed strand because of a central termination sequence that prevents full-length +DNA synthesis. This results in a single-stranded DNA flap. Although the mechanism is not known, the DNA flap is needed for efficient entry into the host cell nucleus (Arhel, Souquere-Besse, & Charneau 2006). The cPPT was added to the transfer plasmid of many later generations of *lvv* to improve transduction efficiency of non-dividing cells (Sirven et al. 2000).

1.4.5 *Nuclear import*

Once reverse transcription is completed, the complexes are regarded as being integration-competent (Nisole & Saib 2004) and termed the pre-integration complex (PIC). In 2006 an elegant method of labeling HIV integrase (IN) was reported (Arhel et al. 2006). Insertion of a small tetracysteine tag (CCRECC) onto the C-terminal of HIV-1 IN allowed labeling of the RTC/PIC with the Fluorescein Arsenical Helix binder (FlAsH). Images of the translocation of the RTC/PIC through the cell were obtained. There was evidence for cytosolic translocation along both microtubules and the actin cytoskeleton. The paper then reports an attachment to the nuclear

membrane that they refer to as docking, and while occasional intranuclear IN molecules were visualized, the mechanism or route of entry was still unknown.

While gammaretroviruses only infect dividing cells because they require the cell to undergo mitosis to access the nucleus, lentiviruses can enter the non-dividing cell nucleus. It is thought that transcription factors bind LTR enhancers in the nucleus before integration, synergizing with IN in tethering retroviral PICs to enhancer-containing domains of transcriptionally active chromatin (Felice et al. 2009). Lentiviral entry via direct disruption of the nuclear membrane by accessory protein Vpr has been proposed (de Noronha et al. 2001) but recently a report of direct binding of IN with the nucleoporin NUP153C domain strongly suggests nuclear entry is via interaction with the nuclear pore complex (Woodward et al. 2009).

1.4.6 *Integration*

Integration of the reverse transcribed dsDNA viral genome into host chromosomes is an important step in the retroviral life-cycle because it ensures that the integrated provirus will be replicated when the cell divides and will be present in both daughter cells.

The viral IN protein is key for integration. It is a 32kDa cleavage product of the gag-pol polyprotein and although the exact crystal structure is yet to be solved, it is known to contain three main domains (Jaskolski et al. 2009). The zinc binding domain contains an HHCC putative zinc finger motif (Burke et al. 1992) that promotes multimerisation of IN thus increasing enzymatic activity (Zheng, Jenkins, & Craigie 1996). The catalytic central domain contains the D64-D116-E152 motif, commonly called $DX_{39\text{-}58}DX_{35}E$ or DD35E (Ellison et al. 1995; Shibagaki & Chow 1997) while the DNA-binding domain resides at the C-terminus (Mumm & Grandgenett 1991).

Two catalytic steps are required for integration; both are catalysed by IN. Cleavage and removal of a dinucleotide adjacent to the att sites at the end of the viral

DNA (Katzman et al. 1989), known as 3' processing is essential for subsequent integration into host DNA in the nucleus, but actually occurs within the PIC in the cytoplasm (Chen & Engelman 2000). The second catalytic step, known as "joining" or "strand transfer" happens in the nucleus. IN catalyses a nucleophilic attack by the free 3'-hydroxyl group of the viral DNA on host DNA phosphodiester linkages. The 3' viral ends are ligated to the 5'-O-phosphate residues of the target DNA in a transesterification reaction (Bushman & Craigie 1991).

The final integration steps involve removal of unpaired dinucleotides from the 5' end of the viral DNA and repair of single-strand gaps between the viral 5' end and the target DNA. These steps are probably performed by cellular factors (Van & Debyser 2005). The integrated viral genome is commonly termed the provirus.

Interestingly, it was known as early as the mid-1970's that non-integrated (extrachromosomal) viral DNA can also exist in the nucleus of retrovirus-infected cells as linear or circular DNA molecules (Gianni, Smotkin, & Weinberg 1975). The circular DNA molecules can form via homologous recombination between the two LTRs (giving rise to one-LTR circles) or via non-homologous end-joining, producing two-LTR circles (Ju & Skalka 1980). After it became apparent that these circular, extrachromosomal viral genomes could support transcription and contribute to the production of viral proteins, IN-defective HIV-1-based *lvv* were developed that are able to deliver the transgene to the nucleus but unable to integrate the transgene into the host chromasomes (Vargas, Jr. et al. 2004;Yanez-Munoz et al. 2006). The advantages and disadvantages of non-integrating vectors will be discussed below.

1.4.7 *Retroviral transcription and nuclear export*

Once integrated in the host genome, the provirus relies on host cellular machinery for transcription. The *cis*-acting elements that interact with the host transcriptional machinery are present in the viral LTR. Transcribed by host RNA

polymerase II, the full-length genomic RNA transcript starts at the 5' end of the R sequence of the 5' LTR. Transcription terminates at the 3' end of the R sequence of the 3' LTR thus generating the viral RNA genome flanked by the R sequences.

In simple retroviruses such as MLV, transcription is regulated by host factors alone so viral RNA levels depend on cell cycle and differentiation stage. Transcription of complex retroviruses such as HIV are also regulated by viral proteins such as *Vpr* (Kino et al. 2002) and Tat that can phosphorylate host RNA polymerase II, causing it to be more active (Parada & Roeder 1996) as well as binding the *trans*-activation response region (TAR) present in the LTR.

Host mRNA molecules are modified by 5' capping, 3' polyadenylation and excision of introns by splicing. Unspliced molecules are normally being retained in the nucleus. Retroviral RNA is also 5' capped and 3' polyadenylated but full-length genomic RNA must be exported for packaging into virions as well as spliced transcripts for translation of viral proteins. Retroviruses have evolved a number of mechanisms that allow nuclear export of incompletely spliced viral RNA. Simple retroviruses contain sequences such as the constitutive export element (CTE) on the 3' UTR of Mason-Pfizer monkey virus that interacts with host Transporter associated with antigen processing (TAP) to directly export the viral RNA (Ernst et al. 1997). Complex retroviruses have evolved additional export mechanisms (Harris & Hope 2000). HIV-1 utilises the Rev-RRE system in which the Rev protein recruits the nuclear export factor RanGTP (Fornerod et al. 1997) via interactions with importin 1 (Neville et al. 1997) to the incompletely spliced RNA facilitating export via the nuclear pore complex. It has been shown that addition of the CTE to HIV can facilitate Rev-independent nuclear export (Wodrich & Krausslich 2001).

1.4.8 *Translation of viral proteins*

Once in the cytoplasm, viral RNA can be used for both packaging into virions and as a template for translation of viral proteins (Butsch & Boris-Lawrie 2002). The viral mRNA species in the cytoplasm are heterogeneous as many

retroviruses including HIV contain splice sites that are designed to be inefficient (Staffa & Cochrane 1994). All viral transcripts are templates for translation including full length genomic RNA the translation of which generates the gag and gag-pol polyproteins. Readthrough of the stop codon is achieved differently by different retroviral strains. During translation of MLV genomic RNA the ribosome can mis-read the amber stop codon as a glutamine (Yoshinaka et al. 1985). Ribosomal frame-shift is used by a majority of retroviruses including HIV. In this case the ribosome "slips" back a single nucleotide and contiguously translates pol (Jacks & Varmus 1985).

Env is translated from a separate reading frame. In HIV, Env is produced as a result of leaky scanning from mRNA that contain *env* as the second or third reading frame (Schwartz et al. 1990). Following oligomeric assembly and transport to the golgi compartment, Env is proteolytically cleaved by cellular furin to form TM and SU proteins (Stein & Engleman 1990). Regulatory and accessory proteins, present in complex retroviruses, such as HIV-1, are generated through splicing to produce open reading frames on additional transcripts (Bolinger & Boris-Lawrie 2009).

1.4.9 *Assembly, budding and maturation*

Infectious virions are formed when gag, gag-pol and env assemble together with genomic RNA at the plasma membrane. Present on the RNA is a packaging signal (Ψ). Ψ is a sequence that forms a secondary structure that interacts with gag to facilitate incorporation into the virion. In HIV-1 the Ψ sequence is located approximately 300 nucleotides from the 5' end and is necessary and sufficient to promote RNA dimerization (Darlix et al. 1990) leading to NC-interactions and ultimately incorporation into virions.

Vpr is packed into HIV virions through an interaction with gag (Lu, Spearman, & Ratner 1993) and while Vif and Nef are thought to be incorporated non-specifically, Nef requires myristylation for efficient inclusion (Camaur & Trono 1996;Bukovsky et al. 1997)

The gag polyprotein is largely responsible for virion assembly in HIV. When HIV particles form and bud from the plasma membrane the spherical arrangement of gag proteins associated with the inner viral membrane of immature virions are proteolytically processed by the viral protease giving rise to matrix (MA) nuclear capsid (NC) and capsid (CA) proteins that rearrange to form conical or sometimes cylindrical capsid structures in mature, infectious virions (Ganser-Pornillos, Yeager, & Sundquist 2008). It is this capsid core of the virus particle that is released into the cytoplasm of an infected cell.

1.5 *Retroviral vectors*

Transgene integration into host DNA is the desirable feature of retroviral vectors, however, they have also been shown to be oncogenic due to insertion within or adjacent to proto-oncogenes, a phenomenon known as insertional mutagenesis. The two main types of retroviral vectors have been derived from gammaretrovirus and lentivirus. The advantage particular to lentiviruses is that they can infect non-dividing cells.

Retroviral vectors are designed such that the transgene of interest replaces the coding region of viral RNA genome (*gag-pol-env*) on a plasmid known as the transfer vector. *Cis* elements Ψ, PBS, PPT and viral LTR are still included to facilitate RNA encapsidation, reverse transcription and integration. All proteins needed for vector production are supplemented in *trans* (on separate plasmids). This spacial segregation renders the vector capable of one round of replication only.

1.5.1 *Gammaretroviral vectors*

Gammaretroviral vectors are most commonly based on the Moloney murine leukaemia virus (MLV). In the simplest transfer vectors, the transgene is placed between Ψ and the PPT with transcription being regulated by the enhancer and promoter in the 5' LTR and the polyadenylation signal being provided by the 3' LTR.

The first strategy for providing *gag*, *pol* and *env* genes in *trans* to produce MLV particles used a DNA plasmid encoding the viral genome missing the Ψ sequence (Mann, Mulligan, & Baltimore 1983). While this packaging plasmid was able to drive virus production, after ten days, replication-competent infectious viruses were detected. This was because of recombination between the transfer vector and the packaging construct. This lead to the development of second-generation MLV-based retroviral vectors.

To greatly reduce the risk of homologous recombination between the transfer vector and the packaging construct, in second-generation gammaretroviral vectors the 3' LTR, the PPT and part of the U3 region on the 5' LTR as well as the Ψ signal were removed from the packaging plasmid (Miller & Buttimore 1986). Further improvements in safety derived from separating *env* onto a third plasmid also allowed the development of pseudotyping by providing an heterologous *env* gene from another virus (Danos & Mulligan 1988).

As discussed below in the section on *Gene therapy in humans*, MLV-based vectors were used in successful clinical trials to treat SCID-X1 (Cavazzana-Calvo et al. 2000;Gaspar et al. 2004). By using pseudotyped gammaretroviral vectors to express a functional copy of the common gamma chain (γc) in haematopoietic stem cells, sustained phenotypic and functional immunological correction was achieved. However, because transgene expression is driven by the viral LTR that contains enhancer elements, proto-oncogenes near to sites of integration were activated in some cases leading to leukemia. The risk of such insertional mutagenesis is reduced by insulating transcriptional activation to within the transgene. Self-inactivating (SIN) vectors utilise internal promoters rather than viral LTRs and can include chromatin insulator elements (Arumugam et al. 2007).

1.5.2 *Self-inactivating (SIN) vectors*

The rationale behind the design of SIN vectors is that by deleting the enhancer element from the U3 region of the 3'LTR on the transfer vector, the U3

will be absent from both LTRs in the dsDNA following reverse transcription. Hence, transgene expression is dependent on the inclusion of an internal promoter that can be carefully chosen. This is believed to increase the biosafety of such vectors by further reducing the possibility for homologous recombination between the transfer vector and the packaging plasmids (Zufferey et al. 1998) and by reducing the chance of enhancing expression of host genes proximal to the integration site. Additionally, it removes the possibility for transcriptional interference between internal promoters and viral LTRs.

SIN gammaretroviral vectors were the first to be described (Yu et al. 1986). A 299bp deletion in the U3 region that contained the enhancer and included the CAAT box was used to eliminate virus-derived promoter activity. An internal inducible promoter was instead used to drive transgene expression.

SIN lentiviral vectors have also been generated in a similar way – by deleting 400bp of the U3 region including the TATA box and leaving just 38bp on the U3 5'end that contain attachment sites for integrase and 18bp from the 3' end (Zufferey et al. 1998). One problem with both types of SIN retroviral vector is that by deleting large sections of the U3 region, the likelihood of transcriptional read-through and thus insertional mutagenesis is actually enhanced (Zaiss, Son, & Chang 2002). This can be reduced be the addition of polyadenylation elements to improve 3' processing (Schambach et al. 2007)

1.5.3 *Lentiviral vectors*

Lentiviruses, able to infect non-dividing cells, have a major advantage over gammaretroviruses for use as gene-delivery vectors as gammaretroviral vectors require cell division for transgene integration into the host genome (Miller, Adam, & Miller 1990). However, because wild-type HIV-1 is a human pathogen that causes aquired immunodificiency syndrome (AIDS), numerous modifications had to be made for the generation of HIV-1-based vectors that are safe for general laboratory use.

The most important safety feature of a retroviral vector system is that it must be replication-defective. A similar approach was used to achieve this for lentiviral vectors as was used for gammaretroviral vectors, namely the segregation of essential viral proteins onto separate plasmids to greatly reduce the chance of replication-competent viruses forming via homologous recombination.

First-generation lentiviral vectors were constructed using a single packaging plasmid that encoded seven of the total of nine HIV-1 proteins, excluding only Vpu, an accessory protein involved in the downregulation of CD4, required for the maintenance of viral infectivity of HIV-1 (Tanaka et al. 2003), and Env, needed for HIV-1 binding and entry to the host cell. The vector instead was pseudotyped with the vesicular stomatis virus glycoprotein (VSV-G) which conveys a broad tropism (Naldini et al. 1996).

It was shown in 1997 that none of the four HIV-1 accessory genes *vif*, *vpr*, *vpu*, or *nef* were required for efficient formation of VSV-G-pseudotyped vector particles (Zufferey et al. 1997). Second generation lentiviral vectors use a packaging construct containing just *gag*, *pol*, *rev* and *tat* and commonly the VSV-G coat protein on a separate plasmid. Third generation vectors have further segregated *rev* to a fourth plasmid and deleted *tat* from the packaging plasmid as the vector plasmid contains a *tat*-independent promoter (Dull et al. 1998).

The transfer vectors used in this study has the deletion in the U3 region of the 3' LTR eliminating promoter activity. It is therefore said to be a self-inactivating

(SIN) vector. A WPRE element is included, which has been shown to increase viral titre (Zufferey et al. 1999) and a central polypurine tract/central termination sequence (cPPT/CTS) element to enhance transduction in non-dividing cells (figure 1.5). Transgene expression is driven by the spleen focus forming virus (SFFV) promoter that strongly promotes expression in many cell types including fibroblasts and cells of myeloid lineage (Baum et al. 1995). The packaging plasmid encodes both *rev* to mediate nuclear export and *tat* to enable transcription in the vector plasmid LTR. This means that these vectors are second generation SIN vectors.

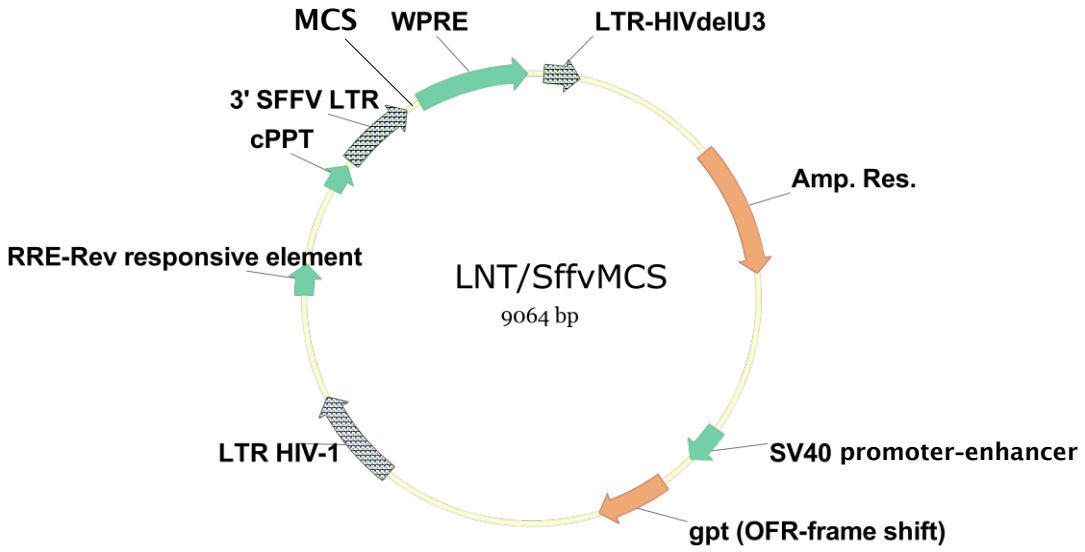


Figure 1.5 Feature map of LNT transfer vector backbone

Features include the internal SFFV promoter, cPPT and a WPRE downstream element that enhances transgene expression. A transgene of interest is inserted in the multiple cloning site (MCS) between the SFFV promoter and the WPRE.

1.5.4 *Integration and insertional mutagenesis*

Although genomic integration of a transgene is advantageous for long-term gene expression in daughter cells when progenitor lineages are transduced, this benefit is balanced by the risk of insertional mutagenesis. The severity of this risk has been illustrated by the occurrence of clonal T cell lympho-proliferative

abnormalities in one patient treated for SCID-X1 in London (Howe et al. 2008) and four in a similar trial in Paris (Hacein-Bey-Abina et al. 2008) both of which used MLV-based vectors. Although it had been suggested that the transgene itself may be oncogenic when overexpressed (Woods et al. 2006) experiments *in vitro* have shown that overexpression of LMO2, a proto-oncogene activated by insertion in four of the five leukemic cases, and not the γc itself that causes aberrant thymocyte growth (Pike-Overzet et al. 2007). The same study shows that of all the T-cell acute lymphoblastic leukemia (T-ALL) oncogenes, LMO2 is most highly transcribed in the CD34+ bone marrow cells and suggests this as an explanation for the preferential targeting of LMO2 over other T-ALL oncogenes.

Experiments designed to assess the risk of insertional mutagenesis derived from *lvv* integration have found them to be safer (Montini et al. 2006) although this study compared SIN *lvv* expressing eGFP with an viral LTR-driven eGFP-based MLV vector. Both were pseudotyped with VSV-G. A similar cell culture assay that compared SIN *lvv* with HIV-1 LTR-promoter vectors and with MLV-based vectors found the SIN vectors to have greatly reduced transforming capacity (Bokhoven et al. 2009). A separate report, however, did find the integration of SIN vectors to perturb expression of adjacent genes in primary, clonal murine beta-thalassemic erythroid cells. Perturbed expression was detected in 28% of the transduced clones. 11% of all genes contained within a 600-kilobase region surrounding the vector-insertion site demonstrating altered expression. This was higher than that observed for a lentiviral vector containing a viral long-terminal repeat (LTR) (Hargrove et al. 2008).

This study uses SIN *lvv* primarily as an experimental tool to achieve sustained transgene expression in cells such as DCs that may not be dividing. Whilst the safety profile of such vectors would be of prime concern if the current strategy was to be translated into the clinic, the present study instead focuses on a method of designing a transgene that induces antigen-specific tolerance. Many vector modifications are under development in labs across the world that attempt to marry

the stability of *lvv*-driven transgene expression with the low toxicities associated with other gene delivery protocols for use in future human trials.

1.5.5 *Gene therapy in humans*

Gene therapy can be defined as the transfer of genetic material into cells for therapeutic gain. The method of transfer, can be physical (gold nano-particles or therapeutic ultrasound), chemical (non-viral vectors such as liposomes), or in the case of viral vectors, biological. Originally intended as a novel treatment for genetic diseases by way of correcting missing or mutant genes via addition of a functional copy, current applications range from the treatment of fatal acquired diseases such as AIDS to gene doping in sport (Trent & Alexander 2006). An index from the Journal of Gene Medicine of all worldwide clinical trials to use gene therapy details over 1,500 past or present trials of which almost 1,000 trials are for cancer diseases.

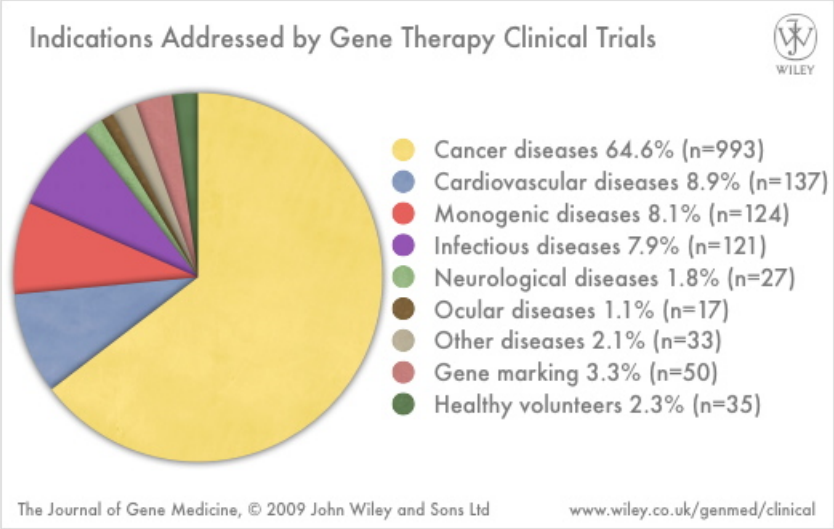


Figure 1.6 Journal of Gene Medicine index of global clinical trials of gene therapy. Data is correct as of march 2009.

The first clinical trial of gene therapy, conducted in 1989 used retroviral vectors to transfer the neomycin resistance gene into tumour-infiltrating lymphocytes that were then infused into five patients with advanced melanoma

(Rosenberg et al. 1990). Whilst the use of neomycin resistance as a marker enabled the group to show that the infused cells infiltrated the tumour and were still present in the tumour after 64 days, the wider implications of the study were that retroviral vectors could be used to introduce a transgene into cells to be administered to human patients and that these cells were able to persist whilst stably expressing the transgene. In 1990 the first therapeutic trial was performed in two girls with a severe combined immunodeficiency (SCID), in this case an inherited metabolic form of the disease called ADA-SCID caused by a mutation in the gene encoding the adenosine deaminase enzyme (ADA) (Bordignon et al. 1995). ADA is involved in purine metabolism, de-aminating both adenosine and deoxyadenosine. In patients with ADA-SCID large amounts of deoxyadenosine accumulate in T cells resulting in a lack of T cell and B cell function (Akeson, Wiginton, & Hutton 1989). Retroviral vectors were again used to transduce T cells, enabling them to produce functional ADA enzyme and thus reduce the patients' dosage of ADA enzyme replacement therapy (Blaese et al. 1995). Similar studies that instead focussed on transducing autologous CD34+ bone marrow (Bordignon et al. 1995) or umbilical cord (Kohn et al. 1998) cells followed, eventually culminating in recent reports in which first one (Gaspar et al. 2006) and then 8 out of 10 patients (Aiuti et al. 2009) to receive such treatment following mild preconditioning chemotherapy were able to completely discontinue costly and potentially immunogenic enzyme replacement therapy.

Other reports of complete or partial cures of human disease have been restricted to hereditary conditions and have numbered a small proportion of the total number of trials undertaken. Our laboratory, simultaneously with a laboratory in Paris, conducted trials on a total of 20 boys with X-linked SCID (Cavazzana-Calvo et al. 2000;Hacein-Bey-Abina et al. 2002;Gaspar et al. 2004). This form of SCID, denoted SCID-X1 is caused by a defect in the *IL2RG* gene, located on the X chromosome that encodes the γc which forms an integral part of cytokine receptors IL-2R, IL-4R, IL-7R, IL-9R, IL-15R and IL-21R resulting in a complete loss of T cells and NK cells (Sugamura et al. 1996). B cells are often present in normal

numbers but are functionally defective (White et al. 2000). By using a gammaretroviral vector encoding the IL2RG cDNA to transduce autologous CD34+ bone marrow *ex vivo*, these cells were re-infused into the patients without any pre-conditioning. The ability of successfully transduced cells to signal through the cytokine receptors listed above confers a survival advantage as they are able to respond to survival signals and other signals. These transduced progenitors reconstitute the bone marrow and give rise to normal populations of T cells and NK cells although there is some variation in this, probably due to patient age at the time of treatment as gene therapy for SCID-X1 has been shown to fail in older recipients (Thrasher et al. 2005).

A very different heritable condition that has been treated with some success by gene therapy is Leber's congenital amaurosis (LCA) (Bainbridge et al. 2008). In this case subretinal delivery of a recombinant Adeno-associated virus (AAV)-based vector was used to deliver human *RPE65* coding sequence driven by a 1400-bp fragment of the human *RPE65* promoter to three patients with defective copies of the gene. Patients who have mutations in the *RPE65* gene experience progressive degeneration of cone photoreceptor cells that ultimately results in the loss of cone-mediated vision because the mutated RPE65 cannot convert all-trans retinol to 11-cis retinol during phototransduction, which is then used in visual pigment regeneration in photoreceptor cells. Although cone photoreceptor cells are responsible for bright-light colour perception, sufferers of LCA experience a prolonged degeneration of visual acuity that often only starts becoming a barrier to leading a normal everyday life in the third decade of life and in dark conditions. The youngest of three patients in this study, a male in his late teens showed significant improvements after six months in his visual function as measured by microperimetry and dark-adapted perimetry and showed vastly improved performance in a dark-condition mobility-test obstacle course. Whilst this alone was an undisputed and widely publisised success, the other two patients in the trial did not display significant improvements, probably in part due to being of a slightly greater age and stage of disease

progression. An application for authorization of a similar study in younger patients is underway (Smith, Bainbridge, & Ali 2009). Another study has used a similar AAV vector to deliver the *RPE65* gene this time expressed under control of a constituitive (chicken β actin) promoter rather than a tissue-specific promoter and the three patients here also showed varied but in one case significant improvements in retinal sensitivity by various measures (Maguire et al. 2008).

In 2005 the Chinese government approved the use of the first comMerck, Nottingham, UKial gene therapy product. Intratumoral injection of Gendicine, a replication-deficient *P53*-expressing adenoviral vector, used in conjunction with radiotherapy to treat head and neck cancers and other solid tumors such as advanced hepatocellular carcinoma, advanced lung cancer and soft tissue sarcoma (Peng 2005). P53 is a tumor suppressor gene that is mutated in at least half of all human malignant tumours (Chen et al. 1999). Treatment with Gendicine with radiotherapy increases life expectancy of cancer sufferers and induces complete remission in 64% of head and neck cancer patients compared to just 19% who received radiotherapy alone (Guangyu Ma et al. 2008).

1.5.6 *Adverse effects of gene therapy*

Even some of gene therapy's success stories have been mitigated with serious adverse events including leukemia and death. The trials that have corrected SCID-X1, generally regarded as the most significant triumph for gene therapy in curing human disease, unfortunately reported leukemia development, as discussed previously in section **1.5.5**. Insertional mutagenesis was located near the T-cell proto-oncogene LMO2. All five children received chemotherapy to treat the leukaemia and whilst four are in sustained remission, one subsequently died after two bone marrow transplants and further chemotherapy.

A protocol similar to that used in these trials by Hacein-Bey-Abina *et al.* in Paris and by Howe *et al*. in London - *ex vivo* transduction of bone marrow using gammaretroviral vectors - has been used to successfully treat the myloid

immunodeficiency X-linked chronic granulomatous disease (X-CGD) (Ott et al. 2006), but one of the three patients in the study subsequently died. This was due to massive bacterial infection followed by colon perforation and subsequent sepsis. Because these complications are known to occur in X-CGD patients, this death is attributed to the underlying disease as opposed to a side-effect resulting from the gene therapy.

In 1999, the first death resulting from gene therapy occurred. In the now famous case of Jesse Gelsinger, a young man with ornithine transcarbamylase (OTC) deficiency that can lead to potentially fatal blood levels of ammonia, he underwent hepatic infusion of an adenoviral vector carrying the OTC gene. Despite high toxicity and morbidity of first generation adeno-vectors in rhesus macaques (Nunes et al. 1999), a dose escalation study of OTC-bearing third generation adenoviral vectors in baboons showed only limited toxicity at the highest doses (Raper et al. 1998). Of the first 17 human patients to receive a liver infusion of this vector, transgene expression was detected in hepatocytes of seven of the patients and only three of these subjects from eleven who had symptoms related to OTC-deficiency showed improved urea-cycle metabolism (although this was non-significant). Jesse, the 18th subject to be enrolled, suffered a fatal reaction that was due to a systemic inflammatory response leading to intravascular coagulation and multiple organ system failure (Raper et al. 2002). This fatal reaction is thought to be due to an immune response to the capsid of the adeno-vector and although this was largely unforeseen, the investigators and regulators involved in planning and carrying out the trial have since accepted some responsibility (Wilson 2009).

In 2007 a 37 year old woman who had received an intra-articular AAV2-based vector as part of a phase I/II clinical trial of gene therapy for arthritis died (Kaiser 2007). The vector expresses a gene product identical to Etanercept, a protein therapeutic used for RA and other autoimmune diseases that consists of the human type II Tumour necrosis factor (TNF) receptor fused to the Fc region of human IgG1. Four months after receiving an initial intra-articular injection of 5×10^{13} viral

particles into her right knee, she received a second injection and became very sick that day with nausea and vomiting followed by diarrhea and abdominal pain. After experiencing fluctuating symptoms for ten days, she was admitted to hospital with a temperature of 39.5°C. Following treatment with antibiotics, antifungals and multiple blood transfusions, and despite renal replacement therapy for acute renal failure, an ultrasound revealed a massive hemorrhage in the left retroperitoneal space that eventually led to compression of the abdominal organs. Life support was withdrawn and the patient died twelve days after admission to intensive care, three weeks after the second viral injection. Postmortem examination found *Histoplasma capsulatum* in the liver, lungs, bone marrow, spleen, lymph nodes, thymus, kidney and brain. Oddly, the pathological examination found no evidence of RA in either knee (Evans, Ghivizzani, & Robbins 2008) suggesting that the gene therapy was successful in treating the arthritis.

These lethal adverse effects could have been caused by the AAV vector itself or the expressed Etanercept transgene. When taking such TNF antagonists, histoplasmosis is a recognised risk factor and the patient lived in an area where *Histoplasma capsulatum* is endemic. She had, however, been using TNF antagonists already for 5 years prior to the gene therapy and histoplasmosis usually occurs within 6 months of TNF antagonist therapy initiation. Nonetheless, it is probable that the patient was infected with the fungus at the time of the second gene therapy injection and that the resulting immunosupression due to reduced TNF signalling resulted in acute infection, eventually leading to mycotic aneurysm, retroperitoneal bleed and death.

Trace amounts of vector genomes in the blood, spleen, liver and brain of the subject, larger amounts in the tonsils and high copy numbers in the right knee were detected by quantitive polymerase chain reaction (qPCR) (Evans, Ghivizzani, & Robbins 2008). *Rep* gene sequences, absent from the recombinant vector were detected in the heart, trachea and right knee, but not in the blood, spleen, liver, brain

or tonsils. This suggests that replication-competent AAV were present although the detected levels were low enough for this to be disputed (Frank et al. 2009).

Despite the cause of death being uncertain, the FDA lifted the hold on this trial in December 2007, and it proceeded with more stringent criteria on enrolling and treating patients, being completed in May 2009. One issue that this death raises is the safety of expressing a non-specific immunosuppressive. Whilst direct administration of a drug such as Etanercept can be ceased in the event of acute infection, expression of such a therapeutic cannot be stopped so easily. As discussed below, all other gene therapy trials for arthritis essentially express anti-inflamatory peptides, either in the form of antagonists or soluble receptors for inflammatory cytokines such as TNF or IL-1, or directly express anti-inflamatory cytokines themselves such as TGF-β. The present study instead demonstrates that an alternative strategy can be to induce antigen-specific tolerance using *lvv* injected *iv*. Rather than restricting the anti-inflammatory effects of transgene expression to the location of inflammation, this approach restricts the anti-inflammatory effect to the antigen of interest.

1.5.7 *Gene therapy for rheumatoid arthritis*

As described above in the case of the Phase I/II clinical trial that used AAV-based vectors to deliver the Etanercept TNF antagonist, the two primary advantages of current clinical trials of gene therapy over directly administered protein therapies are; expression of the protein therapeutic can be physically limited to the inflamed joint and that endogenous expression of a protein therapeutic results in it's prolonged availability eliminating the need for repeat dosing (Evans, Ghivizzani, & Robbins 2006).

Recombinant IL-1Ra, called Anakinra, but also marketed as Kineret (Amgen Biologicals) is administered by daily *sc* injection as an effective treatment for RA (Fleischmann et al. 2006). A protocol for introducing the IL-1Ra cDNA into the joints of RA patients was proposed in 1996 (Evans et al. 1996), and this was the

basis of the first clinical trial for RA gene therapy. Results from this trial were published in 2005 (Evans et al. 2005). Recombinant retroviral vectors expressing cDNA for Anakinra were used to transduce autologous synovial fibroblasts *ex vivo*. These cells were expanded as were untransduced cells. After tests for replication competent virus, endotoxin, mycoplasma, and other adventitious agents were negative, transduced or untransduced synoviocytes were intra-articularly injected in a double-blind fashion into the 2nd-5th metacarpophalangeal joints. 11 out of 12 joints that received the higher doses of transduced synoviocytes did produce elevated levels of IL-1Ra after they were surgically removed on day 7, although this was insufficient to have a therapeutic effect. Publication of these results was delayed by more than 5 years to give a report on longer-term safety aspects. The retroviral vector used was the same MLV-based vector used in the SCID-X1 trials in Paris and London that resulted in leukemia in 5 out of 20 subjects. It was feared that during the week between injection of gene-modified synoviocytes and their removal via surgery because some of the subjects experienced inflammation at the site of injection, some gene-modified cells may have egressed the intra-articular space, escaping surgical removal and then have the potential to subsequently cause adverse effects. There was, however, no clinical or molecular evidence of side-effects and no detectable replication-competent retrovirus.

The first evidence of a clinical response to gene therapy in human arthritis was published in February 2009 using an *ex vivo* transduction protocol based on that described above (Evans et al. 2005) to express recombinant IL-1Ra in autologous synoviocytes (Wehling et al. 2009). In this study, two post-menopausal females under the age of 75 that required a surgical synovectomy of metacarpophalangeal joints 1-3 on both hands were selected. Approval was originally given to treat six patients but due to the report of leukemia as a result of the SCID-X1 trials (Kohn, Sadelain, & Glorioso 2003), the trial was unable to be completed. Results only from the first two patients, treated before approval was withdrawn, was presented.

Synovial fibroblasts from one hand were injected into the joints of the contralateral hand. Again, untransduced fibroblasts were used as intra-patient controls. In this study the transduced synovial fibroblasts remained in the recipient joint for 4 weeks during which the subject kept a "pain diary". Patient 1 showed a dramatic improvement in pain score in both "high dose" treated and "low dose" treated joints as measured by VAS Pain Score. Joint circumference was also found to reduce in patient 1, indicating reduced inflammation, even despite a flare of her RA during the study period, possibly due to cessation of methylprednisolone administration. Patient 2 also responded albeit less dramatically.

One feature of the gene therapy protocol in these studies that was identified for obvious improvements was the time-consuming and expensive nature of performing *ex vivo* transduction (Evans et al. 2005). Subsequent gene therapy protocols for treating RA have concentrated on *in vivo* gene delivery.

Preclinical data showed that by injecting naked DNA plasmids encoding the herpes simplex virus-thymidine kinase (HSV-tk) directly into the joint followed by *iv* administration of ganciclovir (a pro-drug that is phosphorylated by HSV-tk) for three days reduced swelling in the knees of rabbits with antigen-induced arthritis (AIA) (Sant et al. 1998). AIA is an animal model of proliferative inflammatory arthritis where the rabbit is immunised to ovalbumin and arthritis is then triggered by ovalbumin injection into the joint. Histologic examination of the joint 21 days after treatment showed evidence of cytolysis that was confined to the synovial lining cells within inflamed synovium. Based on this approach, Roessler's group initiated a human trial that showed no adverse effect in its first patient but, because it overlapped with the death of Jesse Gelsinger, recruitment was disrupted and the trial was terminated (Evans, Ghivizzani, & Robbins 2009).

Focus has since shifted to using AAV-based viral vectors because of their very low immunogenicity, improved manufacturing protocols (Grieger, Choi, & Samulski 2006) and self-complementary genome configurations that enhance transduction efficiencies (McCarty et al. 2003). A recent finding, however, has

suggested that neutralizing immune reactions to AAV-2 might prevent re-dosing (Kay et al. 2009), a phoenomenon that may have contributed to the massive adverse reaction that lead to the death of a subject in 2007, discussed above in *Adverse effects of gene therapy*. In those trials (a Phase I and Phase I/II, both sponsored by Targeted Genetics Corporation) AAV-2-based vectors delivered the cDNA encoding etanercept, a TNF antagonist fused to the Fc region of human IgG1. The dose-escalation Phase I study found the treatment to be well tolerated (Mease et al. 2009). The Phase I/II trial is now closed but as yet unreported. Work to refine this type of TNF-inhibitory delivery system is underway. By using AAV-5 vector delivering a TNF inhibitor coupled instead to the Fc domain from mouse IgG1 under control of the NF-κB-responsive promoter, a large therapeutic efficacy was observed in the rat adjuvant-induced arthritis model (Adriaansen et al. 2007). In this case, expression of the fusion protein can be "switched off" by NF-κB blockade. Although not as easily controllable as "tet-on" or "tet-off" systems, using a promoter with multiple NF-κB-response elements has the advantage of enhanced expression in cells responding to inflammatory stimulus.

A trial for the use RNAi approaches to reduce TNF-signalling are reportedly underway (Evans, Ghivizzani, & Robbins 2009) as are trials of the use of decoy oligonucleotide NF-κB inhibitors injected directly into the joints of RA patients based on pre-clinical studies by Tomita *et al*. that found such dsDNA to act as decoy cis elements to bind the transcription factor and block the activation of such proinflammatory cytokine genes as (IL-1) and tumor necrosis factor thus reducing the severity of joint destruction.

Although lentiviral vectors have been used in animal trials treating and preventing arthritis (Gouze et al. 2003;Gjertsson et al. 2009), none of the 21 human gene therapy trials currently using lentiviral vectors are for arthritic or autoimmune conditions. Furthermore, none of the previous clinical trials for gene therapy approaches for RA have attempted to induce antigen-specific tolerance to autoantigens implicated in RA pathogenesis.

1.6 *Bio-electrospray*

The work presented below was intended to be an investigation of the use of novel *lvv* for the induction of tolerance by the $CII_{259-273}$ peptide. An important component of the fusion proteins expressed by the novel *lvv* produced in this work is the eGFP domain that allowed intracellular images to be taken using fluorescence microscopy (presented in chapter 4). In the third year of this work a collaborative study was initiated with Dr Suwan Jayasinghe from the Department of Mechanical Engineering, UCL, who was searching for live cells that could be genetically modified and then physically manipulated, being deposited in a controlled way as a droplet or thread, by a process known as bio-electrospray (BES). By transducing 3T3-I-A^q cells with the *lvv* vCII-LAMP, described in this thesis, we were able to demonstrate both that this cell line continued to proliferate following transduction and BES and that they also retained the vector-derived function, namely surface presentation of the $CII_{259-273}$ peptide to MHCII-restricted T cell hybridomas.

BES is a method of using electrostatic charge to control the flow of a cell suspension through a large-bore needle. By applying a voltage to the needle, the cell suspension becomes charged and is attracted to a target cell-culture vessel or solution contained within a beaker attached to a grounded electrode (electrostatically neutral). By varying the flowrate of the cell suspension and the electrostatic potential of the steel, large-bore needle, the cell-structures that are produced in the targed solution can be precisely controlled. By adding 1% poly(ethylene oxide) (PEO) to the solution, we were able to produce cell droplets under high-voltage conditions, and threads of cells under lower-voltage conditions (1-5 kV) (a process known as Cell electrospinning, (CE)). These results are discussed in chapter 6.

Potential clinical applications of such a method of generation of multicellular structures could include the generation of immunomodulatory cell-structures including artificial lymph nodes or other large cell structures that require precise layers or strata of antigen-presenting cells and responder cells. Such structures could

be constructed *in vitro* and implanted at a desired site for particular immunological action, for example within the intra-articular space of an inflamed joint. A second direct application of the method developed in the present study would be to use gene therapy to induce tolerogenic antigen presentation in a population of cells that could be incorporated into a multicellular structure. If this were used to induce autologous iTreg cells *in vitro*, the iTreg cells would be easily separated and re-infused without the need to expose the patient to any potentially carcinogenic gene-therapy-modified cells or tissue.

Chapter 2. Materials and Methods

2 Materials and methods

Table of culture media, buffers and solutions

Buffer/solution	**Recipe/Manufacturer**
4% Paraformaldehyde (w/v) (4% PFA)	4 g paraformaldehyde (SIGMA, Poole, UK) dissolved in 100ml phosphate-buffered saline (PBS)
PBS-tween (PBS-T)	PBS + 0.05% TWEEN20 (SIGMA, Poole, UK)
ELISA/Western blot blocking buffer	PBS-T + 5% BSA
ELISA stop solution	1M Nitric acid
ELISA substrate	R&D Substrate Reagent Pack
Fc receptor block	PBS + 5% heat-inactivated mouse serum
Western running buffer	1 x NuPAGE MES SDS running buffer (Invitrogen, Paisley, UK)
Western sample buffer	NuPAGE LDS sample buffer (Invitrogen, Paisley, UK) + 1 x NuPAGE Sample Reducing Agent (Invitrogen, Paisley, UK)
Western transfer buffer	1 x NuPAGE transfer buffer (Invitrogen, Paisley, UK) made according to manufacturer's instructions

Bacteria culture medium	**Recipe**
LB Broth	20g Luria broth base (Invitrogen, Paisley, UK) + 1 L MilliQ H_2O + autoclaving
LB/Amp selection medium	LB Broth + 100 µg/mL ampicillin (Invitrogen, Paisley, UK)
LB/Amp Agar plates	17.5g LB-Agar (Merck, Nottingham, UK) + 500 mL MilliQ H_2O + autoclaving + 50 mg ampicillin at 50ºC before pouring

2 *Reagents and equipment*

All DNA restriction and modifying enzymes were from Promega, Southampton, UK, unless otherwise stated and oligonucleotides were custom-ordered and generated by Invitrogen, Paisley, UK, UK. All cell culture reagents were from (GIBCO/Invitrogen, UK) unless otherwise stated. Polymerase chain reactions and other thermal cycling protocols were performed on an UNO-Thermoblock (Biometra, Gottingen, Germany). All DNA concentrations were determined by measuring the absorbance at 260 nm using a Nanodrop machine (Thermo, Sussex, UK).

2.1 *Design and generation of fusion constructs*

Initial fusion constructs were cloned into the pFUSE-hIgG4-Fc2 vector (Invivogen, San Diego, CA) that contained a sequence encoding the 20-amino acid IL-2 signal sequence (figure 2.1).

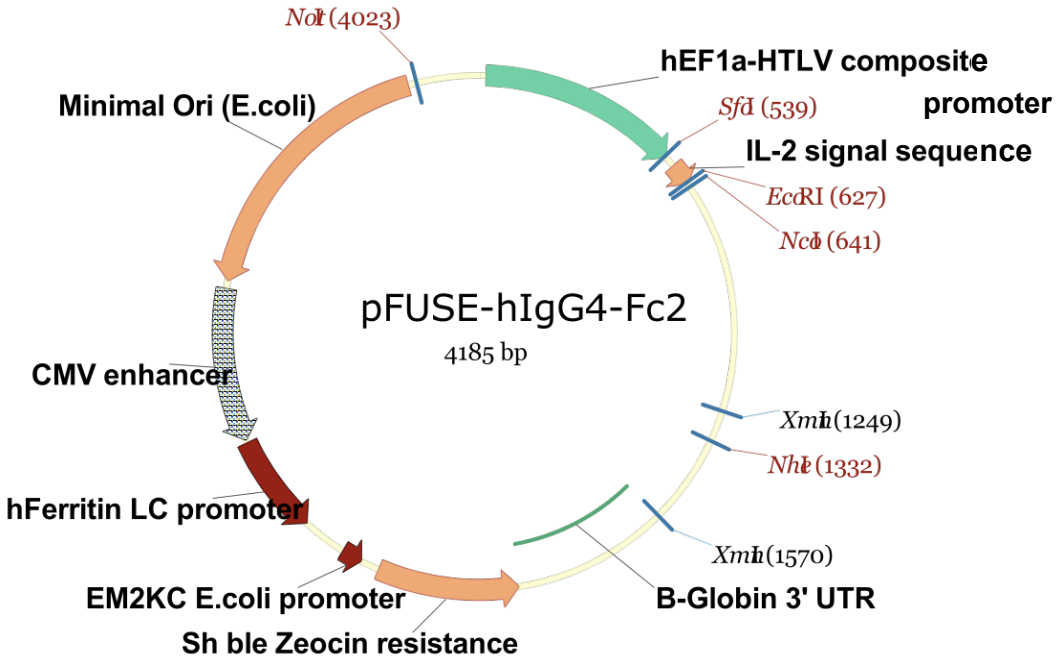


Figure 2.1 Vector NTI map of pFUSE-hIgG4-Fc2.

• hEF1a-HTLV promoter is a composite promoter comprising the human Elongation Factor-1α(EF-1α) core promoter and the R segment and part of the U5 sequence (R-U5') of the Human T-Cell Leukemia Virus (HTLV) Type 1 Long Terminal Repeat. The EF-1α promoter exhibits a strong activity and yields long lasting expression of a transgene in vivo. The R-U5' has been coupled to the EF-1α core promoter to enhance stability of RNA.

• IL2 ss: The IL2 signal sequence contains 21 amino acids and share common characteristics with signal peptides of other secretory proteins. The intracellular cleavage of the IL2 signal peptide occurs after Ser20.

• Minimal Ori (E.coli): A truncated, minimal E. coli origin of replication to limit vector size, but with the same activity as the full-length Ori.

• CMV enhancer / hFerritin LC promoter: This composite promoter combines the human cytomegalovirus immediate-early gene 1 enhancer and the core promoter of the human ferritin light chain gene. This ubiquitous promoter drives the expression of the Zeocin-resistance gene in mammalian cells.

• EM2KC E.coli promoter is a bacterial promoter that enables the constitutive expression of the antibiotic resistance gene in E. coli. EM2KC is located within an intron and is spliced out in mammalian cells.

• Sh ble Zeocin resistance: Resistance to Zeocin is conferred by the Sh ble gene from Streptoalloteichus hindustanus. The same resistance gene confers selection in both mammalian cells and E. coli.

• ßGlobin 3' UTR: The human beta-globin 3'UTR and polyadenylation sequence allows efficient arrest of the transgene transcription5.

The pFUSE-hIgG4-Fc2 plasmid was digested with NcoI and EcoRI by incubating 400 ng DNA with 1 µL 10 X Buffer D and 0.5 µL of each restriction enzyme in a total volume of 10 µL incubated at 37C for 90 minutes. The linear vector fragment was purified on a 1% agarose gel. The EcoRI site is present in the 18^{th}, 19^{th} and 20^{th} codons for the amino acid residues Thr, Asn, Ser of the IL-2 signal sequence. A PCR-amplified eGFP fragment that had also been digested with EcoRI and NcoI was inserted into 100 ng linearised vector with 1 µL T4 DNA Ligase and 1 µL 10 x T4 DNA Ligase buffer consisting of 500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM Dithiothreitol 10 mM ATP, pH 7.5 (Promega, Southampton, UK) in total reaction volume of 10 µL incubated at 18C for 4 hours (below). This ligation conserves the Thr-Asn-Ser amino acid sequence.

2.1.1 *eGFP*

A PCR fragment encoding eGFP with an *EcoRI* site at the 5' end and a sequence encoding the first of two 4-aa sequences that are highly sensitive to cleavage by cathepsin S (Ruckrich et al. 2006) site Leu-Arg-Met-Lys followed by *BamHI* and *NcoI* sites at the 3' end was generated using the polymerase chain reaction:

5 µL 10 mM forward primer 5'-ggaattcgatggtcagcaagggcgaggagctgttcacc-3' and 5 µL 10 mM of the reverse primer 5'-gccatggaaaggatcccttcatgcgaagcttgtacagctcgtccatgccgagagtgatccc-3' (custom ordered, Invitrogen, UK) were mixed with 5 µL 10 X *PfuUltra* HF reaction buffer (Stratagene Inc., La Jolla, CA), 2 units of *PfuUltra* HF (Stratagene Inc.), 0.32 µL 1 µg/µL pEGFP and subjected to a denaturisation step at 95ºC for 5 minutes, 25 amplification

cycles of 58ºC for 1 minute, 72ºC for 2 minutes and 95ºC for 30 seconds followed by a final extension step at 72ºC for 5 minutes. It was decided to use two varying 4-aa sequences reported to be substrates for cathepsin S to increase the likelihood of at least one being efficiently cleaved. The PCR product was cloned into a TOPO-pCRII vector (Invitrogen, Paisley, UK) by mixing 4 µL purified PCR product with 1 µL TOPO-pCRII vector. and subsequently digested with *EcoRI* and *NcoI*. This fragment was ligated into the linearised pFUSE-hIgG4-Fc2 vector by mixing 1 µL linearised vector with 1 µL T4 DNA Ligase, 1 µL T4 DNA Ligase buffer and 7 µL PCR products. Chemically competent MACH-1 T1 phage-resistant E. coli cells (Invitrogen, Paisley, UK) were transformed with the reaction mix. All chemically competent cells were transformed by incubating with 5 µL reaction mixture on ice for 30 minutes followed by 'heat shock' step, 42ºC/30s, ice/1 minute and then incubated at 37ºC for 1 hour before plating onto 25 µg/mL Zeocin plates The daughter plasmid (denoted pG10) was then digested with *NcoI* and *NheI* for insertion of the Lamp1 cytosolic/TM domain (below).

2.1.2 *LAMP-1*

mRNA was extracted from an homogenised mouse spleen using Tri-reagent (Applied Biosystems) and chloroform (VWR). Briefly, a mouse spleen sample was homogenised in 500 µL Tri-reagent, incubated at room temp for 5 minutes and centrifuged for 1 minute before the supernatant was added and mixed thoroughly. After microcentrifugation for 15 minutes, the aqueous phase was transferred to a fresh tube for RNA precipitation and wash using isopropanol (VWR) and 75% ethanol. A cDNA pool was generated using MultiScribe reverse transcriptase (Applied Biosystems) by preparing 10 µL 2X reaction mix, placing on ice and adding 10 µL mouse spleen mRNA sample. The 2 X reaction mix was prepared by adding 2 µL 10 X RT buffer, 0.8 µL 100 mM dNTPs, 2 µL supplied 10 X Random Primers, 1 µL RNase Inhibitor and 1 µL MultiScribe reverse transcriptase to 3.2 µL nuclease-free H_2O. The reaction mix was incubated at 25ºC for 10 minutes, 37ºC for

120 minutes and 85ºC for 5 minutes before incubating on ice breifly. The reaction mix was purified on a QiaQuick column (Qiagen) and RNA was degraded by incubating with RNase H (New England Biolabs, Hitchin, UK) for 30 minutes at 37ºC. Second strand synthesis was performed by using the Klenow fragment (NEB, Hitchin, UK) incubated in 1X NEBuffer 2 for 1 hour at 37ºC followed by heat inactivation at 75ºC for 20 minutes.

A DNA fragment encoding the 48 amino acid residues at the carboxy terminus of mouse LAMP-1 which includes the cytoplasmic tail domain, the TM domain, and a short portion of the luminal domain was cloned using forward primer 5'-accatgggagaatctgaaggggtctgtggaagagtgtgttcag-3' and reverse primer 5'-ggctagcctcgagctagatggtctgatagccggcgtgactcct-3'. The forward primer includes an *NcoI* site and the second Cathepsin S recognition site Glu-Asp-Leu-Lys at the 5' end (Ruckrich et al. 2006), and the reverse primer includes a stop codon, an *XhoI* and an *NheI* site at the 3' end. The 188-bp fragment was cloned into TOPO pCR-II and was subsequently digested out with *NcoI* and *NheI* and inseted into the pG10 plasmid to form pGL1. Restriction endonucleases *BamHI* and *NcoI* were used to open pGL1 between the Cathepsin S cleavage sequences for insertion of the $CII_{259-273}$ peptide (below).

2.1.3 *CII peptide and MOG peptide*

Complementary oligonucleotides encoding collagen residues 259-273 ($CII_{259-273}$) with overhanging ends compatible with *BamHI* and *NcoI* were obtained from Invitrogen, Paisley, UK:

Direct strand 5'-gatccggcatcgctggcttcaaaggtgaacaaggccccaagggagaacctt-3', and complementary strand

5'-gccgtagcgaccgaagtttccacttgttccggggttccctcttggaagtac-3'. These were annealed in 1 x T4 DNA-ligase (Promega) buffer and purified on a 2% agarose gel. The eluted band was resuspended in 10 µL 1 x T4 DNA-ligase buffer and phosphorylated by adding 1 µL polynucleotide kinase (Promega) and incubating at

37ºC for 30 minutes. The reaction mix was gel purified, extracted and serially diluted 100-fold. For ligation into pGL1 (between *BamHI* and *NcoI* sites), 1 µL linearised pGL1 (above) was mixed with 1 µL T4 DNA Ligase, 1 µL diluted phosphorylated oligos and 8 µL H_2O. by mixing to form plasmid pGCL2 (figure 2.2). Plasmid construct sequences were analysed by test restriction digestions (see figure 3.2A) and confirmed by sequencing the fusion construct with internal forward primer 5'-gacgacggcaactacaag -3' and internal reverse primer 5'-gctgttgtagttgtactc-3', complementary to regions of the eGFP sequence, using the Sanger method (MWG Biotech, London).

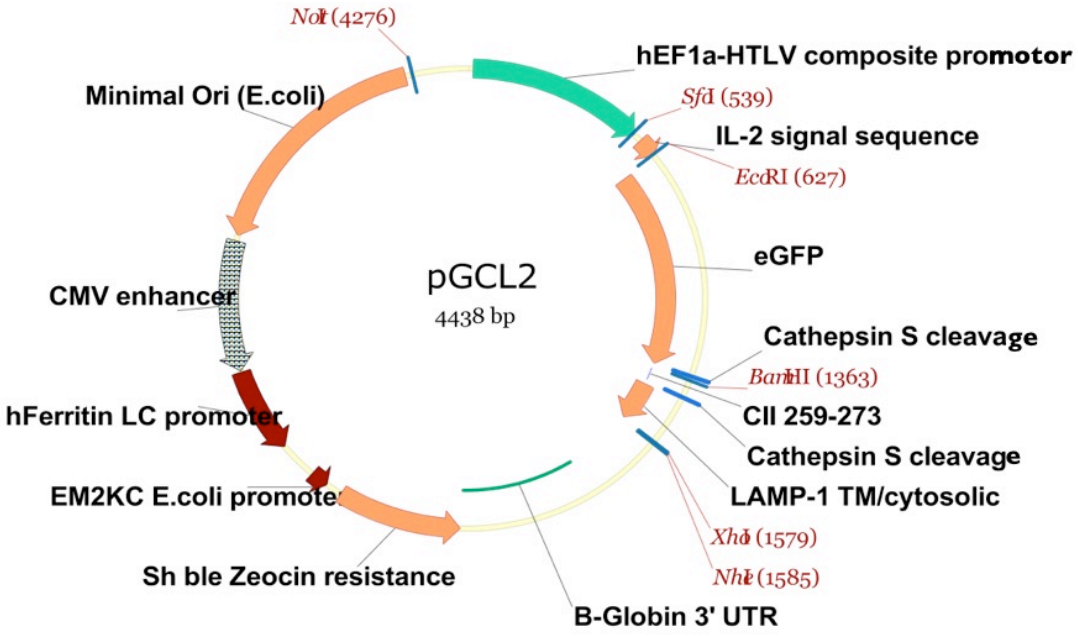


Figure 2.2 Features map of vector pGCL2

• Minimal Ori (E.coli): A truncated, minimal E. coli origin of replication

• hFerritin LC promoter: core promoter of the human ferritin light chain gene

• EM2KC E.coli promoter is a bacterial promoter that enables the constitutive expression of the antibiotic resistance gene in E. coli

• Sh ble Zeocin resistance: Resistance to Zeocin is conferred by the Sh ble gene from Streptoalloteichus hindustanus

• ß-Globin 3' UTR: The human beta-globin 3'UTR and polyadenylation sequence
• LAMP-1 TM/cytosolic: The 188-bp sequence encoding the transmembrane domain and cytoplasmic tail of the mouse LAMP-1 protein
• CII 259-273: Sequence encoding the collagen 259-273 peptide
• eGFP: Enhanced green fluorescent protein

A Myelin oligodendrocyte glycoprotein 79-90 peptide (MOG_{79-90} peptide) control for the *in vivo* experiments was generated in the same way by ordering forward primer 5'-gatccggcaaagtgaccctgcgcattcagaacgtgcgctttt-3' and reverse primer 5'-catgaaaagcgcacgttctgaatgcgcagggtcactttgccg-3', annealing and inserting into the pGL1 plasmid that had been opened with *BamHI* and *NcoI* giving rise to the daughter plasmid pMOG6 that was sequenced with internal forward primer 5'-gacgacggcaactacaag -3' and internal reverse primer 5'-gctgttgtagttgtactc-3' using the Sanger method (MWG Biotech, London).

2.1.4 *H2-DM-targeted fusion constructs*

Chimeric DNA sequences designed to encode fusion proteins targeted to subcellular compartments by the TM/cytoplasmic signal-anchor sequence from mouse H2-DM rather than LAMP-1 were also generated; a DNA fragment encoding the 65 amino acid residues at the carboxy terminus of mouse H2-DMb2 was cloned from mouse spleen cDNA using forward primer
5'-accatgggagaatctgaagagcgggacctctgagcccatc-3' and reverse primer
5'-cgctagcctcgagctagtgccgtccttctgggtagg-3'. The 223-bp fragment that includes the *NcoI* site and cathepsin S cleavage site on the 5' end and a stop codon, an *XhoI* and an *NheI* site at the 3'-end of the reverse primer - as for LAMP-1 (above) - was cloned into TOPO-pCRII, removed by digestion with NheI and NcoI, and ligated into the pG10 plasmid adjacent to eGFP. Annealled, phosphorylated oligonucleotides encoding the $CII_{259-273}$ peptide was then inserted between BamHI and NcoI sites to form pGCH1.

2.1.5 *Insertion of construct into lentiviral backbone plasmid*

Constructs were initially inserted into the Invitrogen, Paisley, UK transfer vector backbone pDEST via the Gateway system. The Gateway system is an off-the-shelf *lvv* backbone-generating procedure that utilises homologous recombination between a 3.8 kb pENTR1A plasmid that contains convenient multiple-cloning sites and a kanamycin resistance gene with the *sffv*- and WPRE-containing lentiviral transfer vector that contains an ampicillin-resistance gene. A transgene of interest is inserted into pENTR1A, selected on kanamycin plates before using Gateway LR Clonase, a recombinase, to catalyse a recombination reaction that transfers the transgene into the pDEST backbone. Prior to recombination, the pDEST plasmid contains the *ccdB* gene that is toxic to E. coli, preventing them from growing due to production of the toxin ccdB that binds to the E. coli DNA gyrase. Hence, E coli harbouring unrecombined pDEST plasmids are unable to grow. When the transgene from pENTR1a has replaced the *ccdB* gene in pDEST these successfully recombined plasmids enable host E. coli to grow.

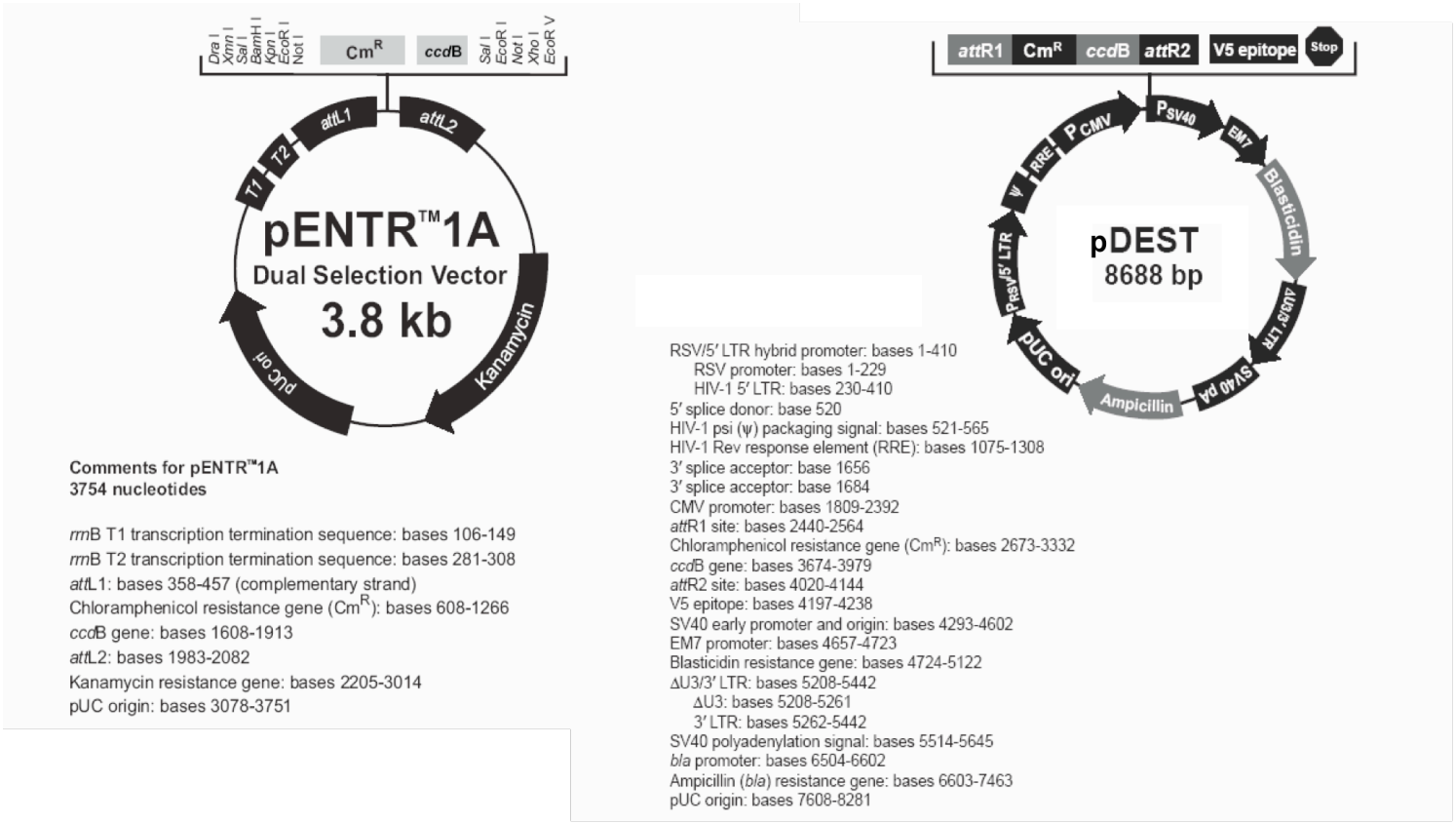


Figure 2.3 Schematic plasmid maps of Gateway Plasmids.
Homologous recombination between attL1 site (pENTR1A) and attR1 site (pDEST) and between attL2 site (pENTR1A) and attR2 site (pDEST) resulting in LR-catalysed transfer of the transgene from the pENTR1A plasmid into the pDEST plasmid in the correct orientation. Figure adapted from www.invitrogen.com.

pGCL2 and pGCH1 were each digested with *SfoI* and *XhoI* to release a ~1 kb fragment comprising the entire construct from the start codon preceding the IL-2 ss on the 5' end to a stop codon on the 3' terminus. This was purified on a 1.5% agarose gel. The fragment was inserted into pENTR1A that had been linearised with *XhoI* and *XmnI* using T4 DNA ligase to form plasmids pGCL8 and pGCH4 respectively. The pENTR1A daughter plasmids includes attL1 and attL2 recombination sites on each side of the gene of interest which recombine with attR sites present on the

pDEST vector to transfer the transgene into the pDEST vector in the correct orientation, downstream from the *sffv* promoter, in a reaction catalysed by the recombinase LR Clonase (Invitrogen, Paisley, UK). Briefly, 200 ng pGCL8 or pGCH4 and 300 ng pDEST vector was reacted using 4 µL LR Clonase in 1X LR Clonase reaction buffer, total volume 20 µL, at RT for 1 hour. 2 µL Proteinase K solution (Invitrogen, Paisley, UK, provided with LR Clonase reaction kit) was added to each reaction to terminate recombination and 2 µL from each reaction was used to transform competent *E. coli* strain DH5α that were plated on LB-agar plates that also contained kanamycin at a concentration of 100 µg/ml. Colonies were selected, amplified and DNA extracted to generate plasmids pGCL12 and pGCH12. Both pGCH12 and pGCL12 were sequenced with internal forward primer 5'-gacgacggcaactacaag -3' and internal reverse primer 5'-gctgttgtagttgtactc-3', complementary to regions of the eGFP sequence, using the Sanger method (MWG Biotech, London).

2.1.6 *Generation of transfer vector with construct expression driven by the hEF1α-HTLV promoter in place of the sffv promoter*

100ng pGCL2 was digested with *NotI*, a unique site ~100bp upstream of the hEF1α-HTLV promoter by incubating at 37°C with 1 µL *NotI* in 1 X Promega reaction buffer D for 2 hours. The sticky end was filled by adding 1 µL 2.5 mM dNTPs and 1 µL T4 DNA polymerase (Promega) and heating at 37°C for a further 5 minutes, before heat-inactivation of the polymerase by heating to 65°C for 20 minutes. After subsequent digestion with *XhoI*, the ~1700bp fragment was purified on a 1% agarose gel, and was inserted into pENTR1A that had been digested with *XmnI* and *XhoI* to generate the plasmid pGCL22 containing the fusion construct behind the hEF1α-HTLV promoter that was then transferred into a pDEST plasmid lacking the *sffv* promoter in a reaction catalysed by LR Clonase as described above to generate transfer vector pGCL29.

2.1.7 *Replacement of the IL-2 signal sequence with LAMP-1 signal sequence*

Multiple attempts to clone the LAMP-1 signal sequence (Lss) from mouse spleen cDNA library were unsuccessful, including nested PCR and 20 combinations of forward and reverse primers. Eventually a set of two pairs of complementary oligonucleotides were ordered that encode the whole Lss sequence in two halves, have an 8-base complementary overhang in the centre and 4-base overhangs compatible with *BamHI* at the 5' end of pair 1 and *EcoRI* at the 3' end of pair 2; forward primer 1: 5'-gatccgtcctccggcctcggctgcgtcgcgccatggcggcccccggcgcccgg-3', reverse primer 1:
5'-agcggccgccgggcgccgggggccgccatggcgcgacgcagccgaggccggaggacg-3', forward primer 2: 5'-cggccgctgctcctgctgctgctggcaggccttgcacatggcgcctcagcactctttgag-3', reverse primer 2:
5'-aattctcaaagagtgctgaggcgccatgtgcaaggcctgccagcagcagcaggagc-3'.

Each pair was annealed, purified on a 100 mL 2% gel agarose gel stained with 1 μL ethidium bromide. Electrophoresis was performed by applysing a potential difference of 100 V with the DNA being loaded into wells placed at the negative-charge end of the gel. Phosphorylation was performed by mixing 9 μL purified, annealed oligos with 1 μL DNA polynucleotide kinase buffer and μL polynucleotide kinase (both Promega, UK). The primer pairs were then ligated in an equi-molar reaction mix (RT, 1 hour) and run directly on a 2% agarose gel. The 117-base Lss was extracted and was then inserted into TOPO-pCRII vector (Invitrogen, Paisley, UK) that had been linearised with *BamHI* and *EcoRI* to form plasmid pLss39.

The pGCL2/pGCH1/pMOG6 *EcoRI-XhoI* fragments (that exclude the IL-2 ss) were then each separately inserted into pLss39 between the *EcoRI* site and a downstream *XhoI* site to generate plasmids LssGCL1 and LssGCH1. Finally these plasmids were digested with *XhoI* and then with *BamHI* for 20 minutes to release the full ~1kb LssGCL or LssGCH construct that was purified on a 1.5% agarose gel and inserted into pLNT/sffvMCS (figure 2.4 A) that had been opened between *sffv* and WPRE with *BamHI* and *EcoRI*.

Insertion of the fusion construct into pLNT/sffv/MCS yielded plasmids pLssGCL12 and pLssGCH12 (Figure 3.8A, B) that were used as transfer vectors on virus preparations to generate lentiviral vectors vCII-LAMP and vCII-DM.

The MOG-peptide-containing construct was inserted into pLNT/sffv/MCS using the same strategy giving rise to vMOG-LAMP. Each of these three final plasmids were sequenced, using the sanger method. The sequences are listed in the appendix at the end of this thesis.

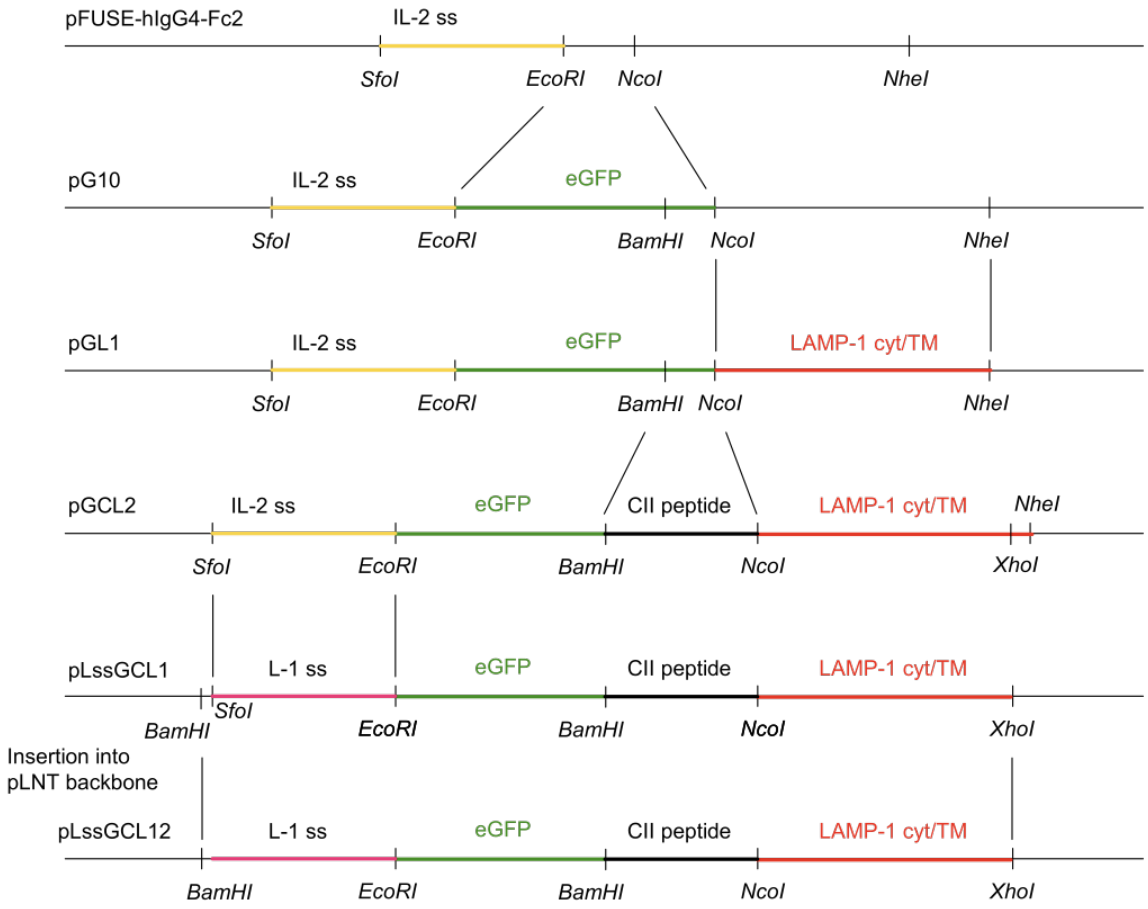


Figure 2.4 A Cloning diagram for GCL constructs. Schematic diagram showing the complete cloning sequence for the final GCL construct, pLssGCL12. LAMP-1 cyt/TM: LAMP-1 cytoplasmic/transmembrane domain, L-1 ss: LAMP-1 signal sequence, IL-2ss: IL-2 signal sequence.

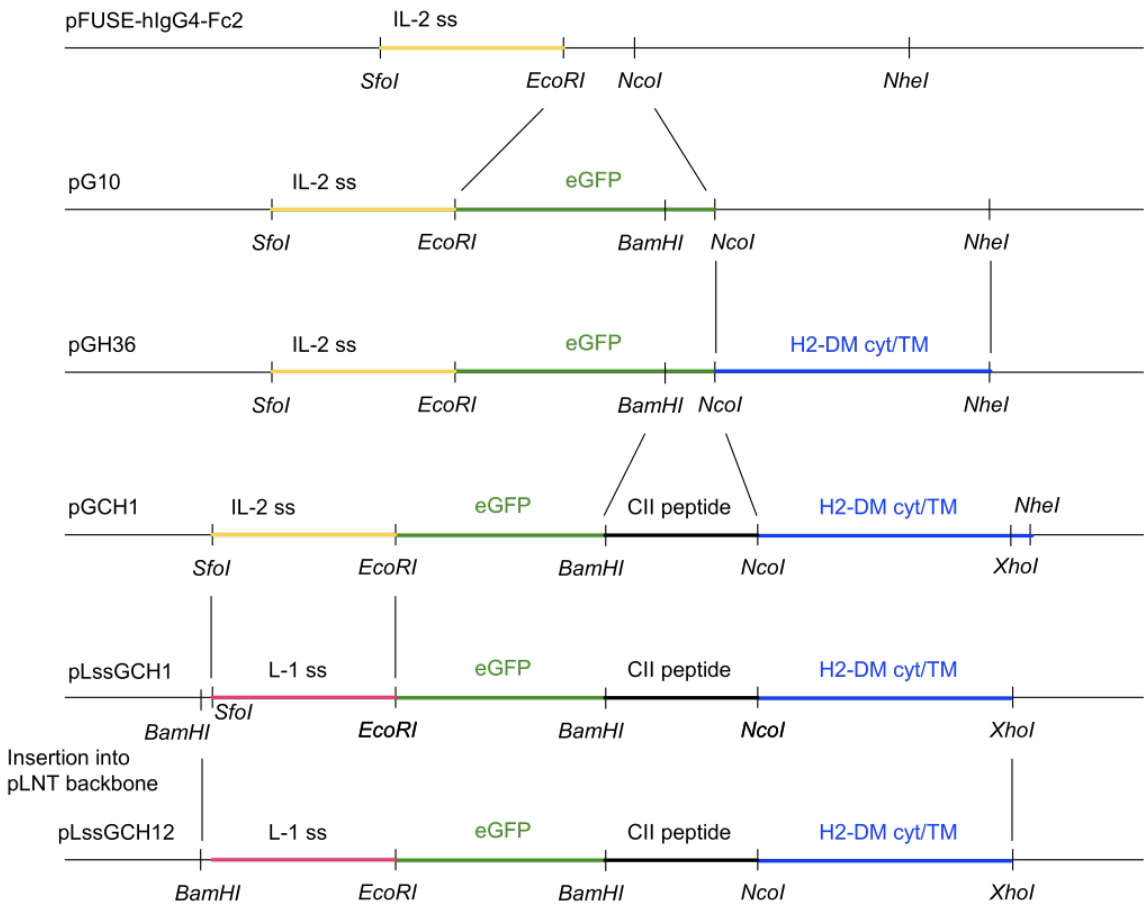


Figure 2.4 B cloning diagram for GCH constructs. Schematic diagram showing the complete cloning sequence for the final GCH construct, pLssGCH12. H2-DM cyt/TM: H2-DM cytoplasmic/transmembrane domain, L-1 ss: LAMP-1 signal sequence, IL-2ss: IL-2 signal sequence.

2.1.8 *Generation of LH3-expressing lvv-backbone*

cDNA encoding LH3 had been cloned, by James Devitt, into a TOPO plasmid from Invitrogen, Paisley, UK but was found after sequencing to contain an

adenine-to-guanine point-mutation that results in a glutamate-to-glycine missense mutation. The single base pair was corrected using a QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

A reaction mix using 2.5 mM dNTPs, 1X reaction buffer, 1X quick solution, 2.5ng/µL forward primer 5'-ggtggctcaagaaggaaatggagaaatatgcagatcagaaagac-3' and 2.5ng/µL complementary primer 5'-gtctttctgatctgcatatttctccatttccttcttgagccacc-3' was set up in a total volume of 49 µL. 1 µL *Pfu* Ultra (supplied with QuickChange kit) was added and the reaction was initiated at 95ºc for 1 minute before cycling between 95ºc for 50 seconds, 60ºc for 50 seconds and 68ºc for six minutes for a total of 18 cycles before finishing the reaction at a temperature of 68ºc for eight minutes. A control reaction was performed without the addition of *Pfu* Ultra, and both reactions were digested with *DpnI* prior to transformation of competent XL10-Gold cells (also provided).

Approximately a dozen colonies were present on the reaction plate and no colonies were present on the control plate. Sequencing of the resulting plasmid preps revealed that the single-base missense mutation had been reversed. The corrected LH3 cDNA was then cloned into the *Xho-BamHI* sites of pLNT/sffv/MCS giving rise to plasmid pLH3cor63.

2.2 *Generation and titration of lvv's*

2.2.1 *Preparation of Lentivirus*

293T cells were grown in DMEM medium with 10% Fetal bovine serum (FBS) in 12 x 275 cm2 tissue culture flasks (Nunc, Roskilde, Denmark) to a density of approximately 80%. Transfection solution was made by adding 13 µL 10 µM polyethylenimine (PEI) to 65 mL Opti-MEM, which was then passed through a 0.22 µm filter (Millipore, Bedford, MA, USA), and split into three 21 mL aliquots, any excess being discarded. Separately, 200 µg of either pGCL12, pGCH12, pLH3cor63 or the GFP-expressing control vector SEW, along with both 70 µg pMD.G2 (Plasmid Factory) that encodes VSV-G and 130 µg pCMVdR8.74 (Plasmid Factory, Bielefield, Germany) that encodes the packaging proteins was added to 20 mL Opti-MEM and filtered through a 0.22 µm filter, added to one of the 20 mL PEI/Opti-MEM aliquots and left at room temperature for 20 minutes with occasional agitation. After removal of cell medium, cells were washed with Opti-MEM and incubated in 10 mL transfection solution at 37°c, 5% CO2. After four hours, the transfection medium was removed and replaced with 17.5 mL DMEM-10% FBS. This medium was harvested and replaced on day 2 and harvested again on day 3. Harvested medium was centrifuged at 4000rpm for 10 minutes, passed through a 0.22 µm filter and virions were harvested by ultracentrifugation in a Surespin rotor (Sorval) at 23,000 x g.

Pellets were resuspended in 200 µL Opti-MEM, frozen in aliquots denoted vLssGCL12, vLssGCH12, vLH3cor63 and vSEW respectively and stored at -80°c.

2.2.2 *Titration of lentivirus preparations by Flow Cytometric analysis of GFP+ cells.*

293T cells were seeded on a 48-well plate (Nunc) at a density of 100,000/ well and were treated with 10 µL of freeze-thawed virus prep or 10 µL of 10-fold serial dilutions in Opti-MEM. On day 3 cells were trypsinised and passed through a

flow cytometer machine (Dako Cytomation CyAn ADP flow) to assess the number of transducing units per mL of virus prep (figure 3.9A). Only values for cell populations of between 5% and 50% GFP+ were used for calculations to reduce the number of cells that had been multiply transduced.

2.2.3 *Titre of LH3-expressing lvv by qPCR – DNA extraction*

Lentiviral vectors that express the lysine-modifying enzyme lysyl-hydroxylase 3 (LH3) were generated as described above using the transfer vector pLH3cor63. Because this *lvv* does not express a fluorescent protein infectious titre could not be ascertained by simple flow cytometric analysis of transduced cells. One approach that has been widely used was to assay the amount of HIV P24 protein in a lentivirus prep, but while this gives a measure of the number of viral particles present, it cannot give a reliable measure of infectious titre due to the possibility of "empty" or otherwise non-infectios virions. Quantatitive PCR, however, provides a direct measure of the number of transgenes integrated in the target cell DNA.

293T cells, seeded in 48-well plates at 100,000 per well, were treated with 10-fold serially diluted vLH3cor63 at a top concentration of a full 50 µL aliquot of frozen-thawed virus prep. On day 3 DNA was extracted by "salting out". Briefly, on day 3 cells were washed with PBS and lysed using 10-µL 1 mg/mL proteinase K (Merck, Nottingham, UK, Darmsdatd, Germany) in 750 µL 8 µM Tris-HCl pH8.2, 0.32M NaCl, 1.87 µM EDTA at 37ºc overnight. On day 4, 200 µL 6M NaCl was vigorously mixed with each sample in a 1.5 mL microcentrifuge tube, before spinning at 13,000 RPM for 10 mins. Supernatant was transferred to a 15-mL falcon tube and 2-mL 100% ethanol was added to precipitate DNA which was pelleted by spinning at 4,600 for 30 minutes. Pellets were washed in 70% ethanol, airdried and dissolved in dH_2O overnight at 37°c.

2.2.4 *Measurement of integration number by qPCR – reaction set-up*

Determination of transgene copy number was performed by qPCR on extracted DNA – both by using WPRE-complementary primers to determine transgene integration number and also mouse β-actin-complementary primers to determine cell number. 25 μL reactions were set up by mixing 20 μL β-actin reaction mix (1100 μL Universal Master Mix, 19.8 μL β-actin forward primer, 19.8 μL β-actin reverse primer, 4.4 μL β-actin probe, 440 μL H_20), or 20 μL WPRE reaction mix (1100 μL Universal Master Mix, 19.8 μL WPRE forward primer, 19.8 μL WPRE reverse primer, 4.4 μL WPRE probe, 440 μL H_20) with 5 μL extracted DNA. Sequence copy number was determined on an Abi Prism 7000 machine using Sequence Detection software.

2.3 *In vitro protocols for analysis of novel fusion proteins*

2.3.1 *Transfection of fibroblasts with pGCL2 and GFP-only controls*

Aliquots of 10 µL lipofectamine (Invitrogen, Paisley, UK) in 90 µL Opti-MEM were prepared and left at room temperature (RT) for 45 mins before addition of 100 µL 5pg/µL, 10-pg/µL, and 20 pg/µL pGCL2 in Opti-MEM were each mixed with 100 µL lipofectamine solution and left at RT for a further 15 mins with occasional agitation. The resulting 200-µL solution was added to 50,000 3T3 fibroblasts adhered in a 48-well plate and washed with Opti-MEM, incubated for 4 hours at 37°c, 5% CO2 before cell medium was added again.

2.3.2 *Western Blot analysis of expressed constructs*

100,000 293T cells, stably transduced with vLssGCL12, vLssGCH12, vLH3cor63 or vSEW at MOI 25 were harvested using a cell-scraper three days post-transduction and heated at 98°c for 10 mins in 1 x NuPAGE sample buffer + reducing agent and run on a NuPAGE Novex Bis-Tris 4-12 % gel alongside NuPAGE Plus2 pre-stained standards (Invitrogen, Paisley, UK). Samples were transfered to 0.45 µm Immobilon-P polyvinylidine difluoride membrane in an XCell II Blot module run at 30V for 1 hour. The membrane was blocked in 2% BSA/PBS overnight at 4°c.

Expression of fluorescent proteins was detected by incubation with rabbit polyclonal anti-eGFP at a 200-fold dilution in 1% BSA/PBS, 0.05% Tween for 2 hours with shaking. Expression of LH3 was detected via incubation O/N with whole rabbit anti-LH3 serum (Ruotsalainen et al. 2006a) diluted 1:10 in 1% BSA/PBS, 0.05% Tween.

After 4 washes in 1% BSA/PBS, 0.05% Tween, the membrane was incubated with HRP-conjugated donkey anti-rabbit IgG (GE, Buckinghamshire) for 2 hours with shaking and, following four more washes, developed by mixing 1 mL each of TMB Substrate Reagents A and B (BD Biosciences), incubating on the membrane

for 30s before removing excess with an absorbent towel and viewing a 10 minute exposure under a UVIdoc gel documentation system (UVItec, Cambridge, UK).

2.3.3 *Confocal Microscopy of 293T cells*

Slides were prepared to view the pattern of eGFP expression within the stably transduced 293T cells. After adherence to polylysine slides (VWR), 1 hour, 37°c, 5% CO2, cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 15 minutes, gently rinsed with PBS and then simultaneously blocked and permeablised with 1%BSA/PBS, 0.1% Triton 100 (SIGMA, Poole, UK), stained with Rhodamine-phalloidin (Molecular Probes, Invitrogen, Paisley, UK), that binds to and stains the actin cytoskeleton, diluted 1:100 in 1%BSA/PBS, 0.1% Triton 100 and washed 3 x PBS, 0.1% Triton 100, and once in water before mounting the coverslip. All confocal images were captured using a Leica SP confocal microscope.

2.3.4 *Generation of Mouse Dendritic cells*

Bone marrow was gently extracted from both tibia and fibula of the rear legs from female DBA/1 mice, sacrificed using CO_2, using a 23-gauge needle and 1 mL syringe. A single-cell suspension in serum-free RPMI was obtained by gently repeatedly passing the cell suspension through the needle, and finally straining through a nylon cell strainer. After centrifuging the cell suspension at 13,000 rpm for 5 minutes at room temperature, the pellet was resuspended in red blood cell lysing buffer (SIGMA, Poole, UK) and incubated at 37°c for 5 minutes. 45 mL PBS was added and following another spin at 13,000 rpm for 5 minutes at room temperature, cells were seeded at 1 x 10^7 each into three T75 tissue culture flasks in 10-mL RPMI supplemented with 10% FBS, 20ng/mL rmGM-CSF, and were incubated at 37°c with 5%CO_2. 20ng/mL rmGM-CSF was also added and refreshed every 3 days.

Where these mBM-DCs were to be transduced they were treated with *lvv* at a multiplicity of infection (MOI) of 20-25 on day 3, standardised on infectivity of

293T cells as measured by FACS analysis of 293T cells transduced by limiting dilutions of viral preps (see section **2.2.2**).

2.3.5 *Confocal microscopy of mBM-DCs*

Cells were fixed in 4% paraformaldehyde for 15 minutes before plasma membranes and internal cell membranes were permeabilised by incubating with 0.1% Triton X-100, 1% BSA/PBS at room temperature for 1 hour. Cells were stained with either rabbit polyclonal anti-EEA-1 (ab2900, AbCam, Cambridge, UK) or rabbit polyclonal anti-LAMP-1 (sc5570, Santa Cruz Biotechnology, Heidelberg, Germany) at 1:100 in PBS/0.1% Triton X-100/1% BSA, with or without biotinylated goat anti-eGFP (Rockland, Gilbertsville, PA, USA) at 1:250 for 1 hour at room temperature. After 3 gentle washes with 0.1% Triton X-100 in PBS, all cells were stained with a mixture of goat anti-rabbit-Cy5 (Invitrogen, Paisley, UK) and streptavidin-FITC (Becton-Dickinson) in PBS/0.1% Triton X-100/1% BSA for 1 hour at room temperature. Following another 3 gentle washes with 0.1% Triton X-100 in PBS, slides were washed with dH2O and cover slips were mounted using Vector Shield (Vector Laboratories, Burlingame, CA, USA) that also contains DAPI nuclear stain. Cells were imaged using a Leica SP confocal microscope.

2.3.6 *Antigen presentation by 3T3-I-A^q cells to CD4+ T cell hybridomas*

The 3T3 mouse embryonic fibroblast cell line had been previously stably transfected with the MHCII-DR I-A^q molecule by Dr K Laurie (denoted 3T3-I-A^q cells)(Gjertsson et al. 2009). These cells were used as APCs in the initial investigation of $CII_{259-273}$-presentation by cells expressing CII-LAMP and CII-DM. 3T3-I-A^q were transduced with vCII-LAMP or vCII-DM at an MOI of 50. Untransduced 3T3-I-A^q cells, 3T3-I-A^q cells stably expressing CII-LAMP, CII-DM, eGFP or the Ii-CII construct were seeded in 48-well plates at an initial density of ~50,000/well and left for 2 days. Untransduced 3T3-I-A^q cells were either treated with 100 μg/mL whole rat CII at the time of seeding, or were treated with CII

peptide 259-273, either unglycosylated, or with an O-linked galactose to hydroxy-lysine in the 264 position, at 10 μg/mL directly before addition of the T cell hybridomas. T cell hybridomas HDBR1, HCQ3, HCQ4, and HCQ10, each specific for MHCII-I-Aq in complex with CII epitopes in various states of glycosylation were thawed and grown in DMEM/10% FBS for four or five days at 37º C, 5% CO_2 before addition to ~ 100,000 3T3-I-Aq cells at a ratio of 1:1. Most experiments were conducted in triplicate. Cells were co-cultured in 48-well plates for 2 days, frozen and thawed and the medium assayed for IL-2 concentration by sandwich ELISA using mouse IL-2 ELISA DuoSet (R&D Systems, Minneapolis, MN, USA) on Nunc Maxisorp 96-well plates. 100 μL standards were prepared by 2-fold serial dilutions of a 2000ng/mL high-standard.

After incubation with detection agent, streptavidin-conjugated HRP at a dilution of 1:1000, substrate solutions A and B were mixed 1:1 and 100-μL added to each well after 3 thorough washes. The colour reaction was stopped by addition of 50 μL 1 M HNO_3. Optic density was measured by subtracting the absorbance at 450 nm from the absorbance at 540 nm using a FLUOstar Optima microplate reader (BMG Labtech, Aylesbury, UK).

2.3.7 *Antigen presentation by mBM-DCs to CD4+ T cell hybridomas*

100,000 primary mBM-DCs, prepared as described above in section 2.3.3, were transduced or treated with rat CII on day 3 and co-cultured with 100,000 hybridomas HCQ3 or HCQ4 on day six. Following two days' co-culture, IL-2 response was measured by sandwich ELISA as described above.

2.3.8 *$Cll_{253-273}$ presentation in cells coexpressing LH3*

150,000 3T3-I-Aq fibroblasts were seeded into each well of a 12-well plate and incubated in 2 mL DMEM - 10% FBS overnight at 37°c with 5% CO2. Cells were washed in Opti-MEM prior to transduction or treatment with rat CII in 100 μL Opti-MEM; 10 μL vCII-LAMP (MOI = 50) or 20 μL vMOG-LAMP (MOI = 50)

either with or without 10 μL vLH3cor63 was added to the transduction wells and 10 μL 5mg/mL native rat CII dissolved in 0.1 M acetic acid to the control well. 20 μL OPTI-MEM was added to the mock-transduced well. After 4 hours incubation at 37°c with 5% CO2 2 mL DMEM supplemented with 10% FBS was added to each well.

2.4 *Assay of protective action of lvv in mouse collagen-induced arthritis*

2.4.1 *Preparation of lvv for use in vivo*

Viral vectors intended for mouse injection were prepared exactly as described above in section 2.2.1 except that the virus pellets were resuspended in 1 mL PBS prior to freezing at -80º. Shipment to the Department of Rheumatology at the University of Gothenburg was made on dry ice using DHL world delivery service.

2.4.2 *Injection of lvv into DBA/1 mice*

Male DBA/1 mice aged 7-10 weeks were used for the *in vivo* experiments and were maintained 10-to-a-cage under standard conditions at the animal facility at the Department of Rheumatology at the University of Gothenburg. All animal manipulations were performed under a local ethical permission and according to the local ethical rules for animal experimentation at the University of Gothenburg.

Administration of vCII-LAMP or vMOG-LAMP was by tail vein injection 28 days prior to CIA induction (ie day -28). 5 x 10^6 infectious *lvv*, as measured through flow cytometry of transducd 293 T cells (see section **2.2.2**), were injected into the tail vein in a total volume of 200 µL (diluted in PBS where necessary). Although only this one dosage was used, higher dosages were avoided as immunisation as opposed to tolerisation might occur. Dose escalation studies may form part of future work but were beyond the scope of this study.

2.4.3 *Arthritis induction and evaluation*

The arthritis induction protocol was based on that developed by Rikard Holmdahl (Holmdahl et al. 1985a). On day 0, 28 days after vaccination with vCII-LAMP or vMOG-LAMP, 9 DBA/1 mice in each group were immunised intradermally with 100 µL rat CII emulsified in 0.1M acetic acid (VWR) and complete Freund's advuvant (SIGMA, Poole, UK-Aldrich, Sweden). A second dose

of 100 μL rat CII emulsified in 0.1M acetic acid on day 21 was supplemented with incomplete Freund's adjuvant and administered *i.p.* 21 days after the primary immunisation.

Mice were graded for arthritic score based on previously described observational protocol, originally used to score septic arthritis (Abdelnour et al. 1993) but also used to determine disease severity in CIA (Gjertsson et al. 2009). Similar scoring systems have also been used in other studies of CIA (Hegen et al. 2006) (Milici et al. 2008). Briefly, arthritis was defined as visible erythema and/or swelling of a limb/joint or digit. A score of 1 was assigned to a limb showing mild swelling and/or erythema, 2 for a limb displaying moderate swelling and erythema and a score of 3 assigned to any limb with marked swelling and erythema, allowing for a theoretical maximum score of 18 per mouse. Digits with clear swelling and/or erythema were assigned a score of 0.25. Scoring of severity of arthritis using this method allows direct comparasson of the results obtained with those from the previous study (Gjertsson et al. 2009).

2.5 *Analysis of samples obtained from experimental animals*

Blood samples were taken on day 28 post CII immunisation and immediately before sacrifice. Spleen and lymph nodes were harvested directly after sacrifice.

2.5.1 *In vitro T cell stimulation and proliferation assay*

Single-cell suspensions were obtained by gently pressing the draining lymph nodes or quartered spleens through 70 μm nylon cell-strainer (BD Bioscience, Bedford, MA, USA) in PBS. After incubation with red blood cell lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl 0.1 mM EDTA) (SIGMA, Poole, UK) at 37ºc for 10 minutes, cells were cultured in Iscoves medium at 10^6 cells per mL in 12-well plates. Cells were cultured without stimuli, or in the presence of CII (50 μg/mL) or 1.25 μg/ mL Concanavalin A (Con A). Supernatant was harvested after 72 hours at which time 1 μCi [^{3}H]-thymidine was added to each well. Plates were cultured for another 20 hours before harvest. Each sample was tested in triplicate.

T cell stimulations and thymidine incorporation assays were kindly performed by Inger Gjertsson at the Department of Rheumatology, University of Gothenburg.

2.5.2 *ELISA measurement of cytokines*

Supernatant from the stimulated splenic and lymph node cultures was harvested after 72 hours and frozen in aliquots. IL-17, IL-10 and TGF-β concentrations were assayed using ELISA kits from eBiosciences (San Diego, CA, USA) by following the provided protocol.

2.5.3 *ELISA measurement of mouse serum antibody titre*

Approximately 300 μL tail vein blood was collected on day 28 of mouse experiment 1, seven days after booster injection, and again immediately prior to termination of the experiment (on day 39 for experiment 1, day 42 for experiment 2). After 2 hours coagulation at RT samples were microcentrifuged for 10 minutes at

6,000 RPM. Up to 150 µL serum was harvested and frozen. Following initial thaw, the samples were measured into 30 µL aliquots before re-freezing for later use. Sandwich ELISAs, described below, were performed to determine antibody titre. IgM levels were assayed only using aliquots that had not undergone a second freeze; IgM-assays were performed on samples directly after the primary thaw.

96-well Maxi-sorb plates (Nunc) were coated with 100 µL either 10 µg/mL rat CII in PBS or 2 µg/mL of unconjugated capture antibody specific for IgG2A or IgG2B in 0.5M sodium carbonate-bicarbonate pH 9.6 by incubating overnight at 4°c. Plates were washed 3 times with PBS containing 0.05% Tween-20 and were then blocked for 1 hour at RT using 200-µL 1% BSA in PBS.

Mouse sera, transported back to London on dry ice, were diluted in PBS at the following dilutions for determination of each isotype: IgM: 1:200, 1:1000, 1:5000, 1:25,000; Total IgG: 1:1000, 1:5000, 1:25,000, 1:125,000; IgG2A: 1:1000, 1:5000, 1:25,000, 1:125,000; IgG2B: 1:1000, 1:5000, 1:25,000, 1:125,000.

IgG2A and IgG2B isotype standards were prepared in duplicate at 1µg/mL and serially diluted in 3-fold dilutions to give a standard curve for assays determining total IgG2a/IgG2b levels as opposed to CII-specific antibody titre.

After blocking solution was removed and the plates were washed as described above, 100 µL sample or standard dilution was added per well and incubated at RT for 2 hours. After a further wash, 100 µL biotinylated or HRP-conjugated isotype-specific detection antibody was applied to each plate for 1 or 2 hours:

Biotin-anti-IgM (Pharmingen): 2µg/mL in PBS, 1 hour, RT.

Biotin-anti-IgG (DakoCytomation): 4µg/mL in PBS, 1 hour, RT.

HRP-anti-IgG2A (Pharmingen): 2µg/mL in PBS, 2 hours RT.

Biotin-anti-IgG2B (Pharmingen): 2µg/mL in PBS, 2 hours RT.

Plates incubated with biotinylated detection antibodies were washed as described above and incubated for a further 1 hour with Streptavidin-HRP (Pharmingen) diluted to 1:1000 in PBS. Assays were developed using TMB

substrate reagents kit (BD Biosciences, San Diego, CA, USA). Substrate solutions A and B were mixed 1:1 and 100-µL added to each well after 3 thorough washes. The colour reaction was stopped by addition of 50 µL 1 N HNO_3.

Optic density was measured by subtracting the absorbance at 450 nm from the absorbance at 540 nm using a FLUOstar Optima microplate reader (BMG Labtech, Aylesbury, UK).

2.6 *Bio-electrospray*

3T3-I-A^q cells were transduced with vCII-LAMP at an MOI of 25 three days before passage through a steel needle with internal and external bore diameters of 6 and 8 mm respectively. This was performed both in a "needle control" (nc) where no voltage was applied to the needle and for bio-electrospray the needle was charged to voltages of ±1-30 kV using the FP-30 precision high-voltage power supply (Glassman Europe Ltd, Tadley, UK), drawing a maximum current of 4 mA. In experiments investigating cell-structure generation, cell media was supplemented with 1% polyethylene oxide (PEO), average M_v ~ 300,000 (SIGMA, Poole, UK). Cells were at a concentration of ~10^6/mL and were sprayed down into a beaker of PBS that was being mechanically stirred such that the stream of medium entered the PBS off-centre. Flow rates of 10 nL/s-100 µL/s were used by regulating the syringe-depression speed using a PHD4400 (Harvard Apparatus Ltd., Kent, UK).

For co-culture experiments to determine the ability of transduced, bio-electrosprayed cells to retain T cell hybridoma-stimulating properties, transduced 3T3-I-A^q cells were processed at a voltage of 20 kV at a speed of 50 µL/s directly into a tissue-culture flask, before co-culture with HCQ4 hybridomas on day 1 for 2 days.

2.7 *Statistical methods*

All data was analysed using GraphPad Prism 5.0. Two-tailed Student's t tests were performed on all continuous data; antibody titres and IL-2 titres that can take any value as opposed to ordinal data that can only have particular values. The statistical significance of differences in ordinal data, such as arthritic scores of experimental DBA/1 mice were evaluated by performing two-tailed Mann-Whitney U tests. P values below 0.05 were considered significant.

Chapter 3. Results I

Generation and expression of fusion constructs

3 Generation and expression of fusion constructs

3.1 *Introduction*

The aim of this project was to induce antigen-specific tolerance to the $CII_{259-273}$ peptide by way of driving MHCII-presentation of the $CII_{259-273}$ peptide. To achieve this, novel fusion proteins that were designed to traffic the $CII_{259-273}$ peptide down the endosomal pathway by virtue of an intracellular localization domain were to be expressed by *lvv*. Two such domains were tested for their ability to drive peptide presentation in the context of MHCII, namely the LAMP-1 cytoplasmic/TM domain (the signal-anchor sequence) and the H2-DM signal-anchor sequence.

H2-DM is the mouse homologue of the human HLA-DM protein, the molecular chaperone that catalyses removal of the Ii-derived CLIP domain from the antigen-binding cleft of MHCII molecules and thus loading of peptides for presentation. To perform this task, H2-DM is trafficked to MHCII-containing compartments and so was thought to be an excellent candidate to efficiently deliver the fusion protein to sub-cellular regions relevant to MHCII-loading. LAMP-1 is associated with the lysosomal membrane. MHCII-containing compartments are considered to be lysosome-like organelles (Santambrogio & Strominger 2006) and LAMP-1 has been used by other groups to efficiently target antigens for MHCII-loading (Wu et al. 1995;Bonehill, Heirman, & Thielemans 2005). Subcellular location and antigen-presentation derived from LAMP-1-targeted fusion proteins (CII-LAMP) and H2-DM-targeted fusion proteins (CII-DM) are compared in Chapter 4, whilst this chapter focuses on their generation and modifications that were found to be necessary in order to achieve *lvv*-mediated expression.

3.1.1 *Signal peptides and signal-anchor sequences*

A short, typically 20-24 amino acid signal peptide at the N-terminus is required for translocation to the ER on proteins bound for the endosomal pathway.

Translocation occurs via binding of the SRP to the signal peptide on nascent polypeptides as they are translated by the ribosome that in turn interacts with an SRP receptor in the membrane of the ER. The Ribosome-SRP-SRP receptor complex then directs translation of the nascent protein through a translocon, also embedded in the ER lipid bilayer, that associates directly with the ribosome and channels the emerging polypeptide into the lumen of the ER resulting in a full-length polypeptide that folds within the ER (Wilkinson, Regnacq, & Stirling 1997).

The signal peptides contain a hydrophobic core. Following translation the signal peptide egresses the translocon laterally, becoming anchored in the ER membrane. In the case of soluble or type I TM proteins that are anchored to the membrane by a signal-anchor sequence present at the C-terminus, the signal peptide is then cleaved by signal peptidase (with type II TM proteins anchored by the uncleaved signal peptide at the N-terminus). It is thought that the C-terminal signal-anchor sequence of type I TM proteins such as LAMP-1 and H2-DM determine whether the protein is ultimately destined for secretion or retention within components of the endosomal pathway.

3.1.2 *IL-2 signal peptide and LAMP-1 signal peptide*

The original cloning strategy utilized a plasmid, pFUSE-hIgG4-Fc2, that contained a sequence that encodes the signal peptide from IL-2 immediately upstream from the *EcoRI* restriction endonuclease site of the multiple cloning sequence. As described below, sequences encoding the other components of the fusion construct were inserted into this plasmid such that the IL-2 signal sequence constituted the 5' end of the fusion construct giving rise to DNA plasmids pGCL29 and pGCH12. *lvv* prepared to express proteins derived from such constructs, however, were repeatably unable to induce fluorescence in cells (figure 3.7).

After the exchange of promoter and vector backbone failed to resolve this problem, the IL-2 signal sequence was exchanged for the sequence encoding the

LAMP-1 signal peptide. This enabled *lvv*-mediated expression of fusion proteins detectable by FACS, microscopy and western blot.

3.1.3 *$CII_{259\text{-}273}$ peptide*

The immunodominant epitope in CIA is a T cell determinant derived from collagen type II; a core region corresponding to amino acid residues 260-270. Studies into the immunogenicity and tolerising capabilities of such peptides have used peptides of varying length, ranging from residue 256 to residue 273. The present study has used fusion proteins that incorporate amino acid residues 259-273, consistent with soluble, synthetic peptides that were used as positive controls for MHCII-peptide presentation. Additionally, 4-amino acid cathepsin S proteolysis site Leu-Arg-Met-Lys was encoded immediately preceding the $CII_{259\text{-}273}$ sequence and the second cathepsin S site Glu-Asp-Leu-Lys to facilitate epitope release within MHCII-containing compartments. (However, no control constructs without such proteolysis sites were made, and it is therefore not possible to determine to what extent these improved the peptide processing.)

3.1.4 *eGFP label*

Identification of fusion protein-expressing cells was achieved by fluorescence microscopy and flow cytometry. Fusion protein-expressing cells are fluorescently active because the fusion protein includes an eGFP domain that emits visible (green) light when excited with UV light. This also enabled intracellular imaging of regions of fusion protein accumulation within the cell.

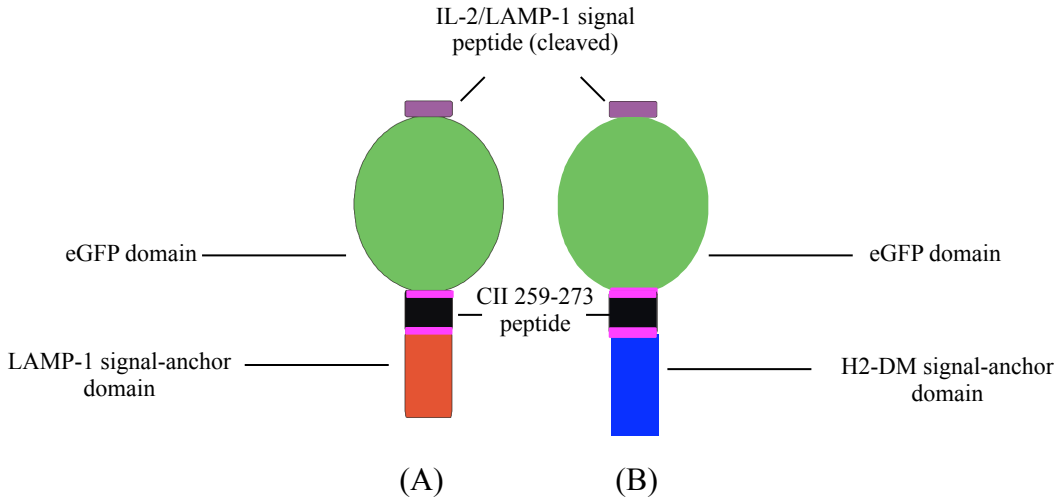


Figure 3.1 Cartoon of fusion proteins (A) CII-LAMP, (B) CII-DM
Schematic cartoon depicting eGFP domain, cathepsin S sites (pink colour), $CII_{259-273}$ peptide and LAMP-1/H2-DM signal anchor domains.

3.2 *Generation of fusion constructs*

Sequences encoding fusion proteins were constructed using standard molecular biology techniques as described in detail in *Materials and methods* and outlined briefly below. Generation of plasmids encoding CII-LAMP is initially described since cDNA for CII-DM was constructed later using equivalent methods (see **3.2.6** *Exchange of IL-2ss for LAMP-1ss and generation of H2-DM-targeted construct*)

3.2.1 *PCR-amplification of eGFP and LAMP-1-signal-anchor domains*

Amplification of eGFP and LAMP-1 signal-anchor sequences flanked by convenient restriction endonuclease sites was achieved by performing a polymerase chain reaction (PCR); custom oligonucleotides that include convenient restriction sites (see *Materials and Methods*) were used as primers on an existing eGFP-containing plasmid template or a mouse spleen cDNA library (used for cloning

LAMP-1, section **2.2.2** above), respectively. Each PCR product was 'cleaned' using a Qiagen kit (figure 3.2)

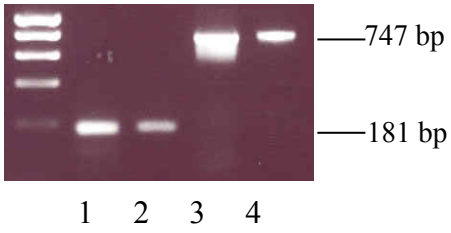
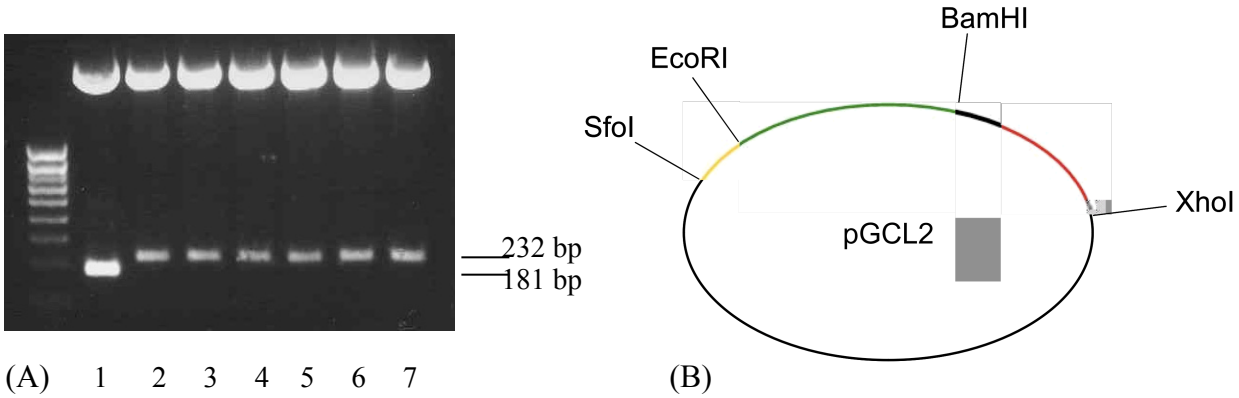


Figure 3.2 Products of PCR to amplify eGFP and the signal-anchor sequence of LAMP-1. Signal-anchor sequence of LAMP-1 before clean-up, lane 1, and after clean-up, lane 2; eGFP cDNA before clean-up, lane 3, and after clean-up, lane 4.

3.2.2 *Assembly of component sequences*

By including an *EcoRI* site at the 5' end of the eGFP forward primer, the clean PCR product (figure 3.2, lane 4) was inserted, in frame, into plasmid pFUSE-hIgG4-Fc2 such that the IL-2 signal sequence was fused to the 5' end, with the hEF1α-HTLV fusion promoter upstream. Other unique restriction sites included on each PCR primer, detailed in *Materials and methods*, were used to insert the LAMP-1 signal-anchor sequence downstream, deriving plasmid pGL1 (figure 3.2A, lane 1). Finally, annealed 51-bp oligonucleotides encoding the $CII_{259\text{-}273}$ peptide including a *BamHI*-compatible overhang on the 5' end and an *NcoI*-compatible overhang on the 3' end were inserted between eGFP and LAMP-1 domains. Successful insertion of the $CII_{259\text{-}273}$ sequence was verified by analytical restriction endonucease digestion with *BamHI* and *XhoI* (figure 3.3A, lane 3), confirmed by DNA sequencing, and denoted pGCL2. Additionally, an IL-2ss-eGFP control construct, denoted pG10 was generated (figure 3.3C) by insertion of an eGFP PCR product generated using a reverse primer that included a stop codon into the pFUSE-hIgG4-Fc2 vector.



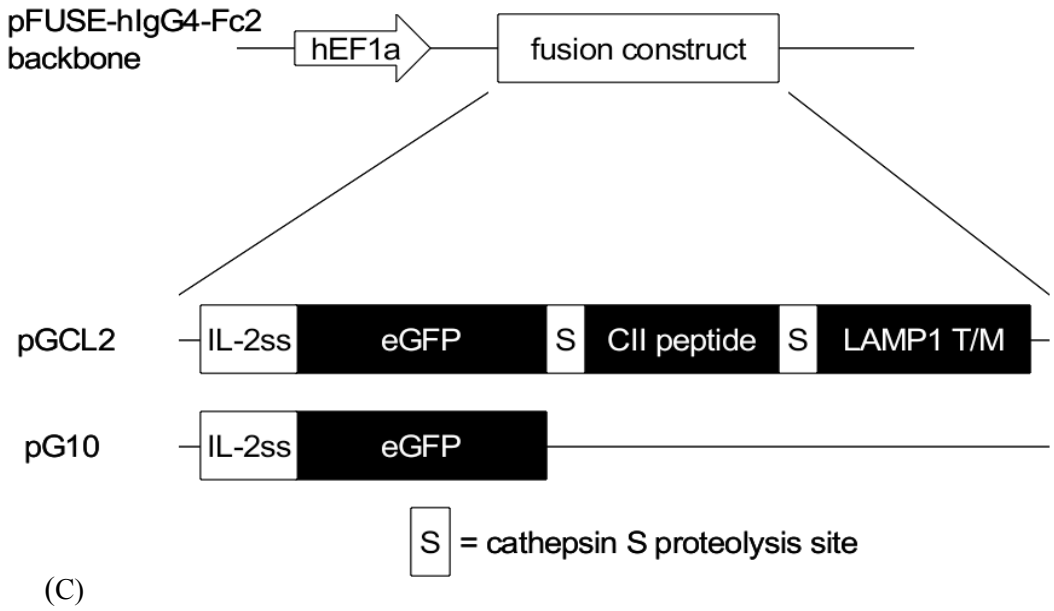
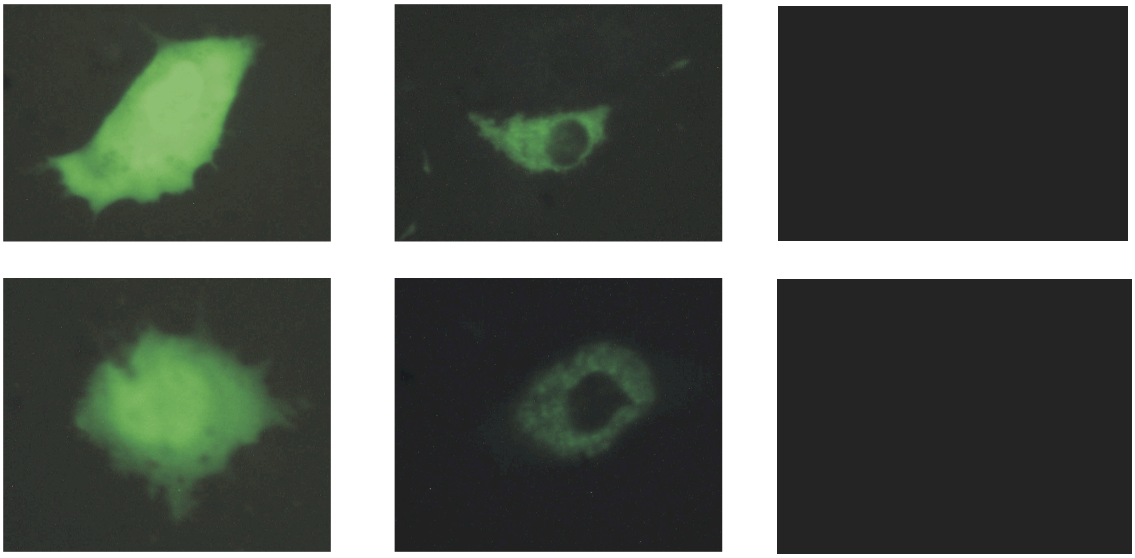


Figure 3.3 IL-2 signal sequence-containing fusion constructs. (A) BamHI-XhoI restriction digestion of minipreps, lanes 2-7, following $CII_{259-273}$ oligonucleotide insertion between eGFP and LAMP-1 domains of pGL1, lane 1. Molecular weight markers correspond to 100-bp, 200-bp, 300-bp, 400-bp, 500-bp, 600-bp, 700-bp, 800-bp and 1 kb. (B) Cartoon of plasmid pGCL2 depicting the IL-2ss (yellow), eGFP domain (green), $CII_{259-273}$ sequence (thick black) and the LAMP-1 TM anchor-peptide sequence (red).

(C) Schematic diagram of fusion constructs pGCL2 and pG10, cloned into the pFUSE-hIgG4-Fc2 plasmid that contains the hEF1α-HTLV fusion promoter upstream of the interleukin-2 signal sequence (IL-2ss) at the 5' end, adjacent to the multiple cloning site into which eGFP, LAMP1 TM and CII peptide/ cathepsin S site sequences were inserted.

3.2.3 *Transient expression of fusion construct following transfection of pGCL2 plasmid*

The pGCL2 plasmid encoding the LAMP-1-targeted fusion construct downstream from the hEF1α-HTLV fusion promoter was transfected into mouse 3T3 fibroblasts using lipofectamine. Fluorescent microscopy was used to visualise eGFP + cells. In cells transfected with pGCL2, fluorescence was visible in a region surrounding the nucleus, whereas in constructs expressing pG10, without a targeting domain, fluorescence is present throughout the cell (Figure 3.4).



(A) (B) (C)

Figure 3.4 Transient transfection of mouse 3T3 fibroblasts. (A) 3T3 cells transfected with eGFP-expressing plasmid pG10 display eGFP throughout the cell, with the brightest region appearing to be within the nucleus; (B) 3T3 cells transfected with pGCL2 display eGFP-fluorescence that appears to be excluded from the nucleus, but within the surrounding regions; (C) 3T3 cells treated with lipofectamine alone. Images taken at 40 X magnification.

3.2.4 *IL-2ss-containing fusion constructs in lentiviral backbone*

The fusion construct including the IL-2ss at the 5' end and a stop codon at the 3' end was excised from the pGCL2 plasmid using *SfoI* and *XhoI* restriction endonucleases and ligated into the pENTR1A backbone that had been digested at *XmnI* and *XhoI* sites located either side of the kanamycin resistance gene. The pENTR1A vector, provided by Invitrogen, Paisley, UK, is designed to recombine by homologous recombination with lentiviral backbone pDEST in determined orientation such that the *sffv* promoter is upstream of the insert in a reaction catalysed by LR recombinase. The presence of the inserted fusion construct in the daughter plasmid pGCL15 was verified by restriction analysis and confirmed by sequencing. However, after 3-plasmids transfection of 293T cells for virus production, cells transfected with the pSEW control vector (expressing eGFP alone) were seen, under UV light, to express eGFP at high levels whereas cells transfected with pGCL12 did not visibly express fluorescent proteins. The virus preparation harvested from these cells was unable to induce visible eGFP-fusion protein expression similar to that seen with transient transfection with pGCL2 in target cells.

Because construct expression had been observed under control of the hEF1α-HTLV promoter following transfection with pGCL2, it was decided that inserting this in place of the *sffv* promoter might resolve the unexpected lack of fusion-protein expression from the lentiviral backbone in the pGCL15 plasmid. The most practical way to achieve this was to insert the entire hEF1α-HTLV-fusion construct cassettes from pGCL2 into pENTR1A and then perform the LR reaction to transfer this into a pDEST plasmid that had had the *sffv* promoter removed.

This two-step procedure was performed and the sequence of the resulting plasmid, pGCL29 was confirmed. The pGCL29 plasmid contains the IL-2ss-containing fusion construct downstream from the hEF1α-HTLV fusion promoter in the pDEST backbone (figure 3.5).

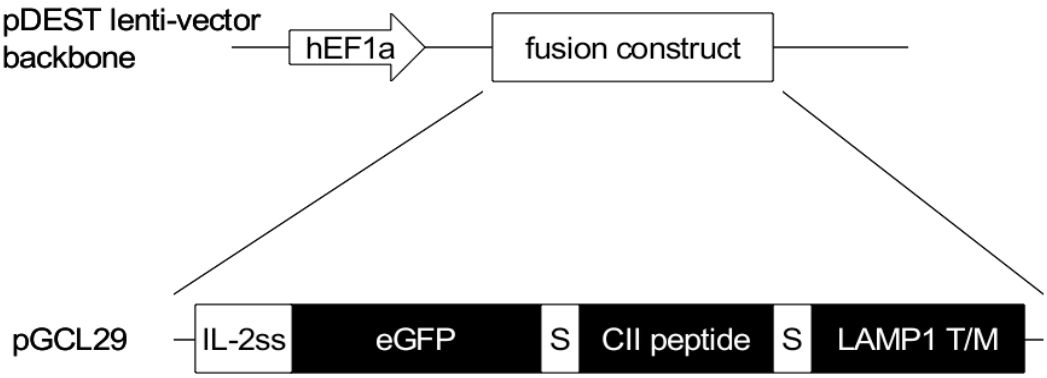


Figure 3.5 Schematic diagram of pGCL29. IL-2ss-containing fusion construct downstream from the hEF1α-HTLV fusion promoter

The plasmid pGCL29 was used along with the packaging and coat plasmids to transfect HEK293T cells to prepare lentiviral vectors as described in *Materials and methods*. The pSEW plasmid, an eGFP-expressing transfer vector was also used to transfect HEK293T cells in separate flasks, again with the packaging and coat plasmids.

Two days after transfection. the 293T cells transfected with pGCL29 cells appeared to be brightly expressing eGFP-tagged construct. These cells were imaged by fluorescence microscopy (figure 3.6). Cells transfected with pSEW also brightly expressed eGFP (data not shown).

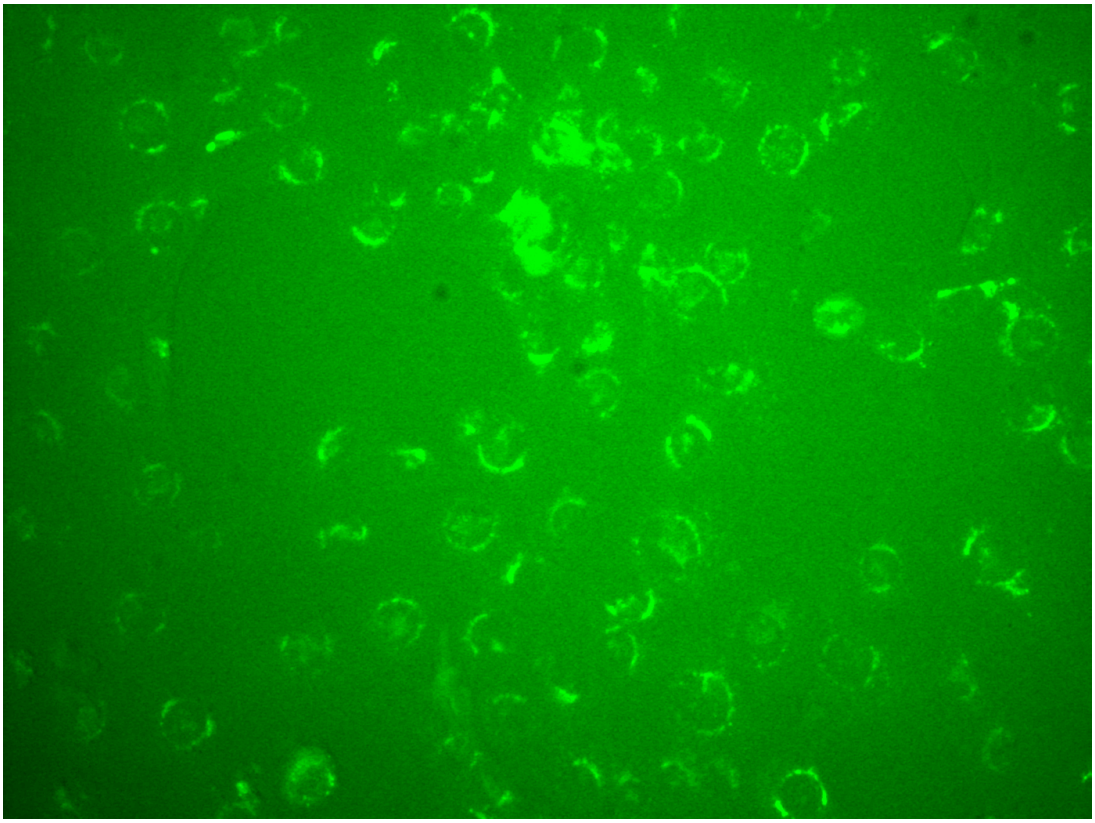


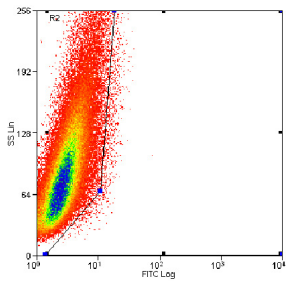
Figure 3.6 Transient transfection with pGCL29 induces fluorescence in 293T cells. Fluorescence micrograph, day 2, of 293T cells transfected with transfer vector pGCL29, VSV-G envelope plasmid pMD.G2 and packaging plasmid pCMVdR8.74. Image taken at 20 X magnification.

Supernatant from the pGCL29- and pSEW-transfected cell cultures were harvested and sterile filtered through 0.22-μm-pore filters to remove cells and cellular debris. Virus particles were purified by ultracentrifugation and frozen in aliquots, and denoted vGCL29 and vSEW, respectively.

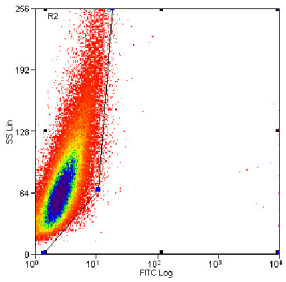
3.2.5 *No expression of fusion proteins was detectable after treatment of 293T cells with vGCL29*

10-μL aliquots of vGCL29 were serially diluted and used to treat 100,000 293T cells growing in 24-well plates. These cells were cultured for several days without any sign of eGFP-fluorescence. Those 293T cells treated with purified virus

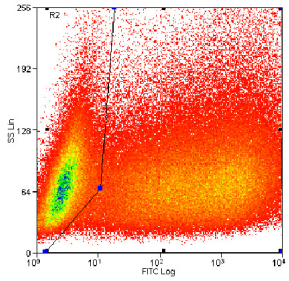
supernatant from the vSEW prep, however, did visibly express eGFP. To be sure that cells treated with vGCL29 prep were not just expressing eGFP-tagged fusion proteins below the threshold of detection with the UV microscope, each cell culture treated with each serially diluted virus-prep sample was run on the flow cytometer (figure 3.7). Measurement of viral titre by transducing 293T cells and determining the proportion of fluorescent cells is the standard method used in the lab for *lvv* that express fluorescent proteins, so this was the method used by the present study.



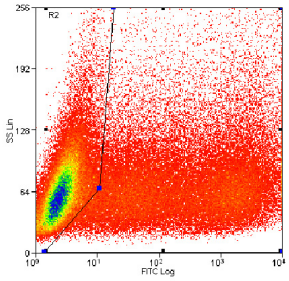
(A) 0.01 µL vGCL29



(B) 10 µL vGCL29



(C) 0.01 µL vSEW



(D) 0.1 µL vSEW

Figure 3.7 Flow cytometric analysis of 293T cells transduced with vGCL29 and vSEW. 100,000 293T cells were treated with 0.01 µL vGCL29 (A), or 10 µL vGCL29 (B). No increase in fluorescence was observed. 293T cells treated with 0.01-µL or 0.1 µL vSEW become 41% eGFP-positive (C) and 62% eGFP-positive (D) by day 2.

Using the 41% positive proportion of 100,000 293T cells treated with 0.01 µL vSEW on day 0, a titre of 4 x 10^9 infectious virions per mL was calculated. Successive attempts to generate virus using pGCL29 yielded no infections titre.

3.2.6 *Exchange of IL-2ss for LAMP-1ss and generation of H2-DM-targeted construct*

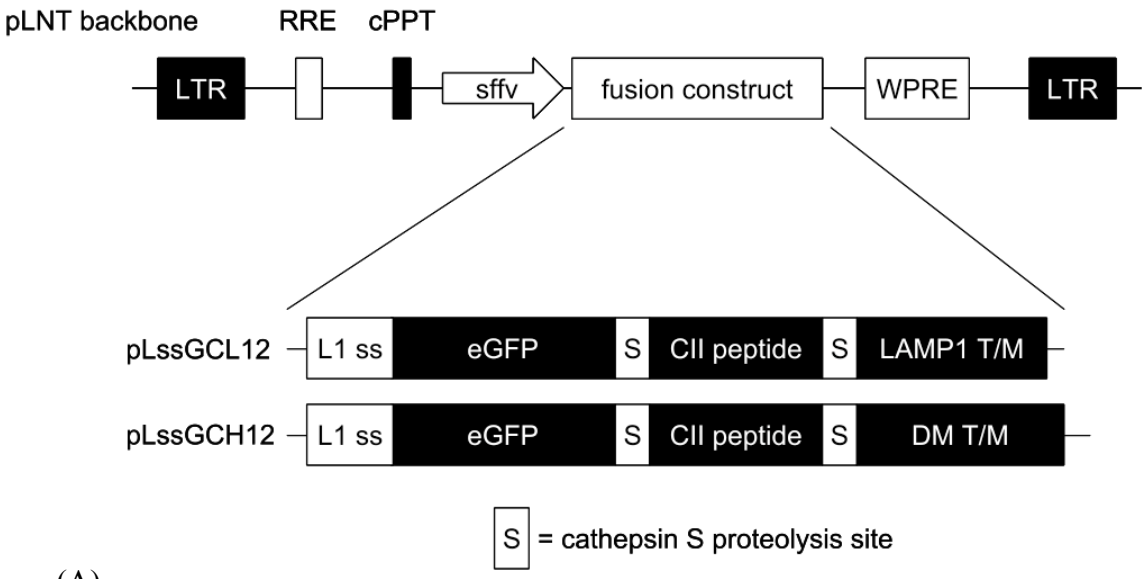
Two approaches to resolve the lack of fusion-protein expression via vGCL29 were undertaken: Firstly, the fusion construct was cloned into a similar but different lentiviral backbone, pLNT. This did not resolve the lack of viral expression, although cloning directly into the *BamHI-XhoI*-linearised pLNT was found to be easier than *lvv*-backbone generation via the pENTR1A-pDEST system. Secondly, two adaptations to the fusion construct itself were made; the exchange of the IL-2 signal sequence for the LAMP-1 signal sequence and replacement of the LAMP-1 TM/cytosolic signal-anchor domain for that from H2-DM.

3.2.7 *Lentiviral expression of LAMP-1ss-fusion proteins*

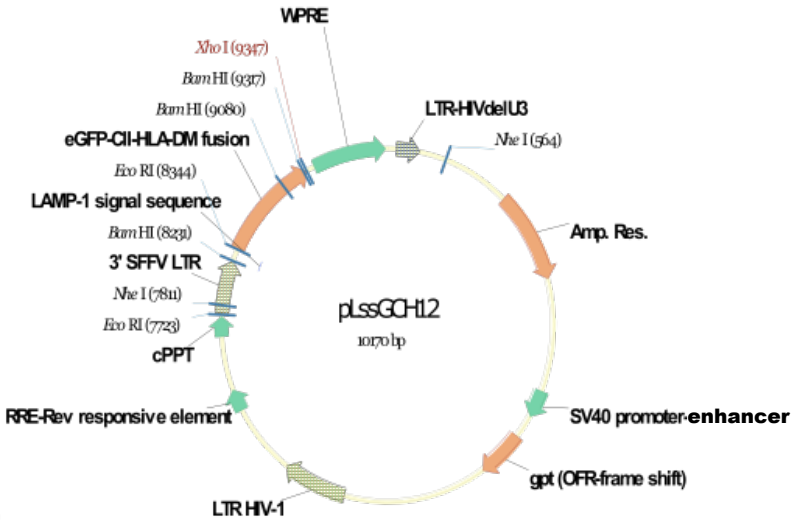
Plasmids encoding constructs that contain the LAMP-1 signal sequence and either the LAMP-1 signal-anchor (pLssGCL12, figure 3.8 A, B) or the H2-DM signal-anchor (pLssGCH12, figure 3.8 A, C) were used to generate virus preps vCII-LAMP and vCII-DM respectively (table 3.1). 293T cells treated with either vCII-LAMP or vCII-DM were found to express eGFP-tagged fusion proteins (figure 3.9 A, B) and this expression was stable over time (figure 3.9 C).

Fluorescent protein	CII-LAMP	CII-DM	eGFP
Plasmid	pLssGCL12	pLssGCH12	pSEW
lvv	vCII-LAMP	vCII-DM	vSEW

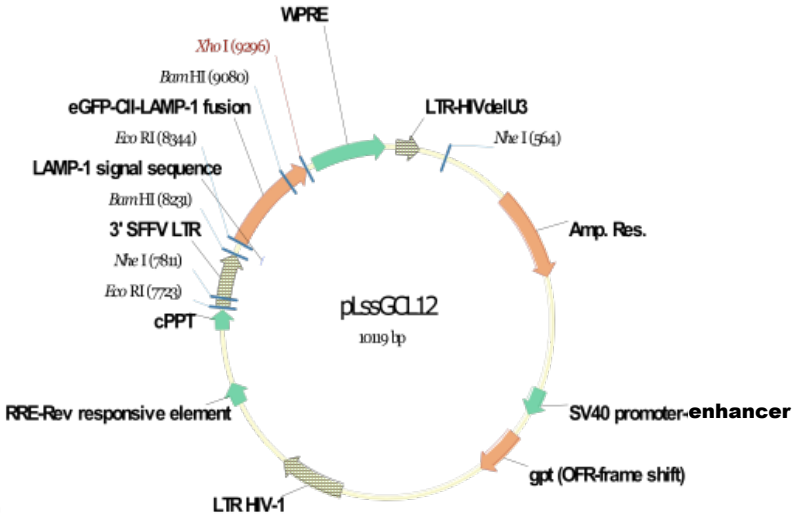
Table 3.1 Summary of novel fusion proteins and their plasmids and lenti-vectors.



(A)



(B)



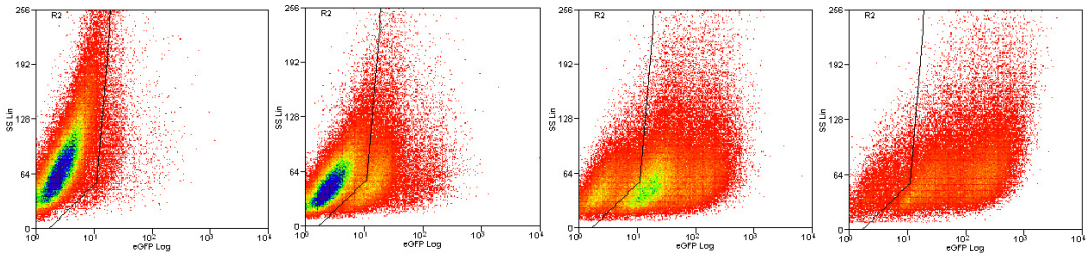
(C)

Figure 3.8 pLssGCL12 and pLssGCH12 transfer vectors.

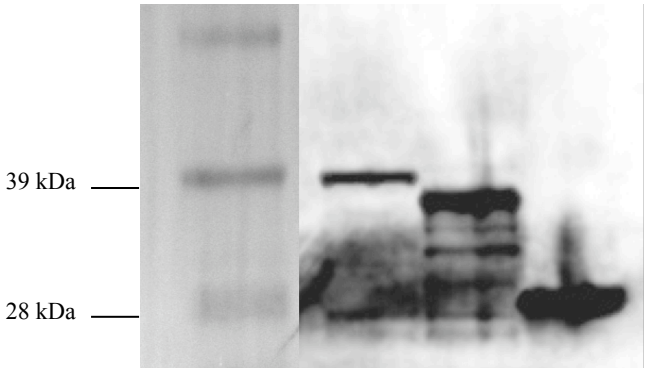
(A) Schematic diagram of pLssGCL12 and pLssGCH12; Rev-response element, RRE; central polypurine tract, cPPT; Woodchuck post-transcriptional regulatory element, WPRE.

(B) Vector NTI map of pLssGCL12

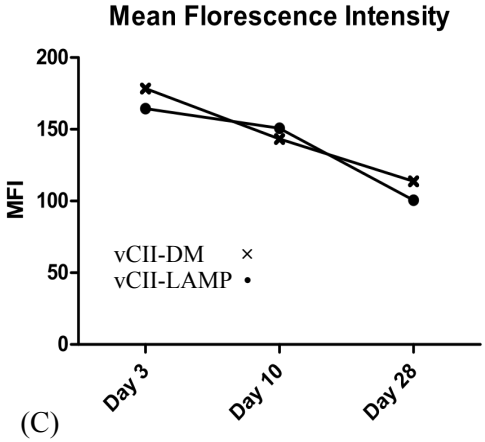
(C) Vector NTI map of pLssGCH12



(A) 0.01 µL vCII-LAMP 0.1 µL vCII-LAMP 1 µL vCII-LAMP 10 µL vCII-LAMP



(B) Std CII-DM CII-LAMP eGFP



(C)

Figure 3.9 Transduced 293T cells fluoresce and western blot reveals degradation of CII-LAMP. (A) Titre of vCII-LAMP by flow cytometry; percentage eGFP-positive 293T cells treated with serial dilutions of vCII-LAMP: 0.01 µL - 3.56%; 0.1 µL - 25.1%, giving a titre of 2.4 x 10^8/mL; 1 µL - 65.3%; 10 µL - 86.9%.

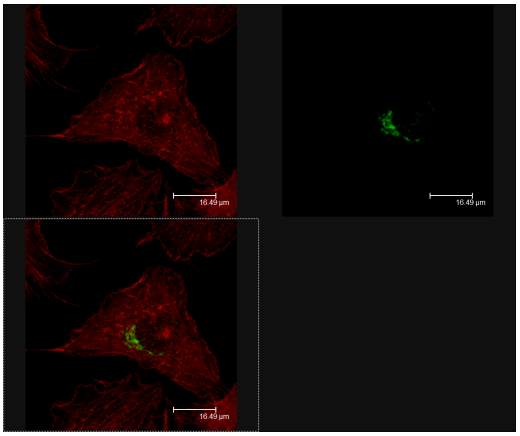
(B) Western blot using anti-eGFP primary antibodies of whole cell lysates from 50,000 vCII-DM, vCII-LAMP and vSEW-transduced 293T cells 3 days after transduction. Pre-stained standards alcohol dehydrogenase (39 kDa) and carbonic anhydrase (28 kDa) confirm sample bands of expected size: CII-DM 37 kDa, CII-LAMP 35 kDa and eGFP 27 kDa and appears to show degradation products for CII-LAMP-expressing cells.

(C) Transgene expression over time. Mean fluorescence intensity (MFI) of 293T cells transduced with vCII-DM (×) or vCII-LAMP (•) was measured on day 3, day 10 and day 28. MFI of vSEW-transduced 293T cells ranges from 800 to 1000 (data not shown).

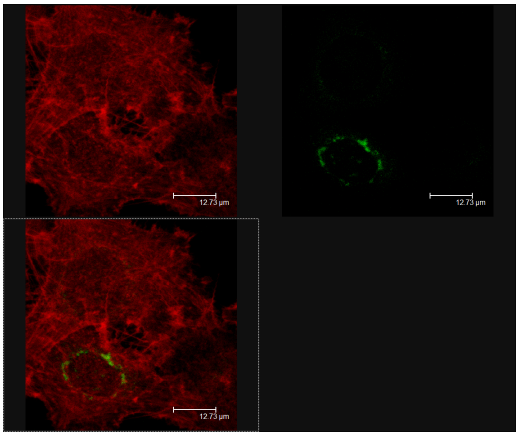
Titres of between 10^8 and 5 x 10^8 infectious particles per mL were consistently obtained for both vCII-LAMP and vCII-DM.

3.2.8 *Confocal micrographs of 293T cells expressing CII-LAMP and CII-DM*

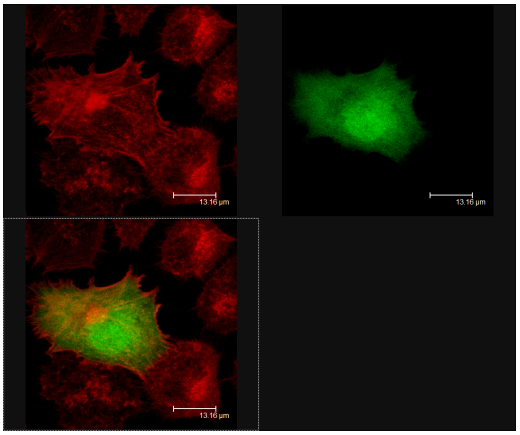
Having shown that vCII-LAMP and vCII-DM were able to induce stable fluorescent protein expression in 293T cells, confocal microscopy was used to image these cells (figure 3.10). Actin filaments were stained with Rhodamine-phalloidin so that the overall extent and shape of the cell could be visualized.



(A)



(B)



(C)

Figure 3.10 Visible fluorescent fusion proteins within transduced 293T cells. (A) CII-DM, (B) CII-LAMP (C) eGFP. Cells imaged on day 3 after transduction and were co-stained with Rhodamine-phalloidin (top-left quadrants) to visualise the actin filaments and thus the structure of the cell. eGFP-fluorescence was visualised (top-right quadrants) and combined (bottom-left). Punctate pattern of eGFP location in panels (A) and (B) contrast with global expression in (C).

3.3.1 *Summary*

The aim of the work presented in this chapter was to demonstrate successful construction of chimeric constructs encoding fusion proteins designed to deliver an immunodominant $CII_{259-273}$ peptide epitope to MHCII-containing compartments. Further, stable expression of these fusion proteins was to be achieved by way of *lvv*-transduction using *lvv*'s that express the fusion constructs.

After successful use of standard molecular biological techniques, fusion constructs encoding CII-LAMP and CII-DM were cloned with the IL-2 signal sequence at the 5' end. Whilst fluorescent fusion protein expression was demonstrated following transient transfection using CII-LAMP- or CII-DM-encoding plasmids, *lvv*'s initially prepared to express these constructs failed to do so. Replacing the IL-2 signal sequence for the LAMP-1 signal sequence resolved this and stable fusion construct expression was shown.

The eGFP domain allowed fusion protein expression to be monitored over time by measuring the mean fluorescence intensity of transduced 293T cells. Additionally anti-eGFP antibodies were used to reveal bands of expected size by Western blot and microscopy revealed subcellular distribution of fluorescent fusion proteins as being distinct from that of eGFP alone. The intracellular distribution of the fusion proteins is investigated further in Chapter 4.

3.3.2 *Discussion*

Detection of eGFP-positive 3T3 fibroblasts after transient transfection was clearly a good indication that eGFP-containing fusion proteins were being expressed by these cells (Figure 3.3) whilst no fluorescence was detected in cells treated with lipofectamine alone. Whilst fluorescence was visible throughout some cells transduced with the IL-2ss-eGFP construct pG10, it is now accepted that the prescence of the IL-2ss on this fusion protein should result in the secretion of the eGFP protein, an outcome that was not investigated but that may have provided an

insight into the reason for non-detection of the IL-2ss-eGFP-$CII_{259-273}$-LAMP-1 fusion protein after transduction with *lvv* generated using plasmid pGCL15.

The observation of degradation products in Western blot analysis of CII-LAMP-expressing cells, but not apparent in samples from eGFP- or CII-DM-expressing cells (figure 3.8) gives a strong indication that the CII-LAMP fusion protein is being processed by proteolysis. This may contribute to the greater efficiency of $CII_{259-273}$-presentation seen in the chapters that follow. Conclusions about the intracellular mechanism behind this data are, however, limited because constructs lacking the cathepsin sites were not generated and cathepsin-inhibitors were not used.

Chapter 4. Results II

In vitro characterisation of fusion proteins

4 *In vitro* characterization of fusion proteins

4.1 *Introduction*

Stable expression of fusion proteins was achieved following modifications of the chimeric transgene as described in chapter 3. Whilst replacing the IL-2 signal peptide allowed *lvv*-mediated expression of CII-LAMP and CII-DM, the rest of the construct was unchanged from the original design that was intended to traffic the protein to MHCII-containing compartments (MIICs). Traffic to MIICs was intended to result in degradation and release of the $CII_{259\text{-}273}$ epitope to allow it to be loaded onto MHCII-I-A^q molecules that it is known to bind. The next question to be addressed was whether expression of these proteins in MHCII-I-A^q–positive cells resulted in cell surface-presentation of the $CII_{259\text{-}273}$ peptide.

Antigen presentation to CD4+ T cells by APCs proceeds after proteolytic processing of material from the endocytic pathway into peptides that may then be loaded onto MHCII molecules. The 3T3-I-Aq fibroblast cell line was initially used to assay vCII-LAMP- and vCII-DM-driven $CII_{259\text{-}273}$-presentaion. Treatment of these cells with either vCII-LAMP or vCII-DM was found to induce $CII_{259\text{-}273}$-presentation. This approach was then extended to primary mouse BM-DCs. Intracellular location of CII-LAMP and CII-DM and their capacity for inducing $CII_{259\text{-}273}$-presentation in mBM-DCs was investigated and compared.

4.1.1 *$CII_{259\text{-}273}$ epitope-presentation to CD4+ T cell hybridomas*

The CII-LAMP and CII-DM fusion constructs were designed such that their expression in APCs should drive presentation of the $CII_{259\text{-}273}$ epitope on MHCII molecules in the following way: An endosomal targeting domain was expected to traffick to the late endosomes where cathepsin S cleavage sites adjacent to the $CII_{259\text{-}273}$ 14-mer were expected to release the peptide epitope, making it readily available to be loaded onto the MHCII molecule by virtue of the cellular components such as HLA-DM. HLA-DMαβ heterodimers have been found localised

to pre-lysosomal and lysosomal compartments due to a YTPL motif on the cytoplasmic tail on the HLA-DMβ polypeptide (Marks et al. 1995). Simillarly, LAMP-1-chimeras are known to localise to MHCII-containing compartments (Arruda et al. 2006). The LAMP-1 and H2-DM chimeras investigated by the present study were designed to improve upon Ii-based chimeras previously investigated by Kenth Gustafsson and colleagues (Gjertsson et al. 2009). To detect and make a comparison between the potency of each *lvv* in driving MHCII-presentation, I-A^q-positive APCs were transduced with vCII-LAMP, vCII-DM or controls and were co-cultured with CD4+ T cell hybridomas that respond to MHCII-I-Aq-bound $CII_{259-273}$ epitope in various glycosylation states. As discussed previously (see **1.2.6** *Posttranslational modifications of CII by enzymes such as LH3 alter the immunogenicity of the immunodominant epitope*), the lysine residue at position 264 can be hydroxylated and further glycosylated and this happens extensively in native collagen. Different CD4+ T cell hybridomas have been previously shown to respond to the $CII_{259-273}$ epitope in particular glycosylation states (table 4.1)(Corthay et al. 1998).

	Rat CII	Naked $CII_{256-270}$	Hyl-264-$CII_{256-270}$	Gal-Hyl-264-$CII_{256-270}$	Glc-Gal-Hyl-264-$CII_{256-270}$
HDBR1	+	+++	++	-	-
HCQ3	++++	-	-	+++++	-
HCQ4	+++	++++	+++	+	-
HCQ10	+++++	-	-	+++++	+

Table 4.1 Summary of CD4+ T cell hybridomas reactive to CII(Corthay *et al.* 1998)**.** HCQ4 is strongly reactive against naked lysine 264 and hydroxy-lysine whilst HDBR1 is listed as being reactive only against hydroxy-lysine although our studies have found it to also react against the naked epitope. HCQ3 and HCQ10 are both strongly reactive to the galactosylated form of the $CII_{256-270}$ peptide.

4.1.2 *3T3-I-Aq fibroblasts as a useful antigen-presenting cell line*

Mouse embryonic NIH/ (3T3-I-Aq) were previously generated by Dr K Laurie in the laboratory and have been used as antigen presenting cells in previous studies of *lvv*-mediated $CII_{259-273}$ presentation that used the invariant chain with the $CII_{259-273}$ epitope exchanged for the CLIP domain (CII-Ii) (Gjertsson et al. 2009). The advantage of using these cells is that as well as stably expressing the MHCII-I-Aq molecule they are robust, fast-growing and easily transduced.

4.2.1 *3T3-I-Aq cells transduced with vCII-LAMP and vCII-DM present $CII_{259-273}$ to CD4+ T cell hybridomas*

Initial experiments assessing the ability of the CII-LAMP and CII-DM fusion constructs to drive $CII_{259-273}$ epitope presentation were performed by transducing 3T3-I-Aq cells with vCII-LAMP or vCII-DM at an MOI of 25 three days before co-culture with CD4+ T cell hybridomas. 3T3-I-Aq cells were also transduced with a CII-Ii-expressing lenti-vector (figure 4.1) to serve as a positive control and with an eGFP- expressing lentiviral vector that served as a vector (transduction) control.

Hybridomas HDBR1, HCQ3, HCQ4 and HCQ10 were thawed and incubated in DMEM/10% FBS medium for 4 or 5 days until the cells began proliferating at which time they are suitable for 2 days co-culture with APCs. Due to varying culture times for different hybridomas before proliferation, the first round of 3T3-I-Aq – hybridoma co-culture experiments only yielded a signal from 3-out-of-4 hybridoma lines (figure 4.1). The hybridoma cell line that proliferates most rapidly, HCQ3, had past the proliferation phase of growth and these cells were largely apoptotic by day 5 when the other hybridomas were ready for co-culture. Hence, no IL-2 was detected in the medium from HCQ3-containing wells (data not shown).

This initial result indicated that CII-LAMP or CII-DM expression in 3T3-I-Aq cells results in $CII_{259-273}$-presentation at levels that strongly stimulate responses from hybridomas HDBR1 and HCQ4.

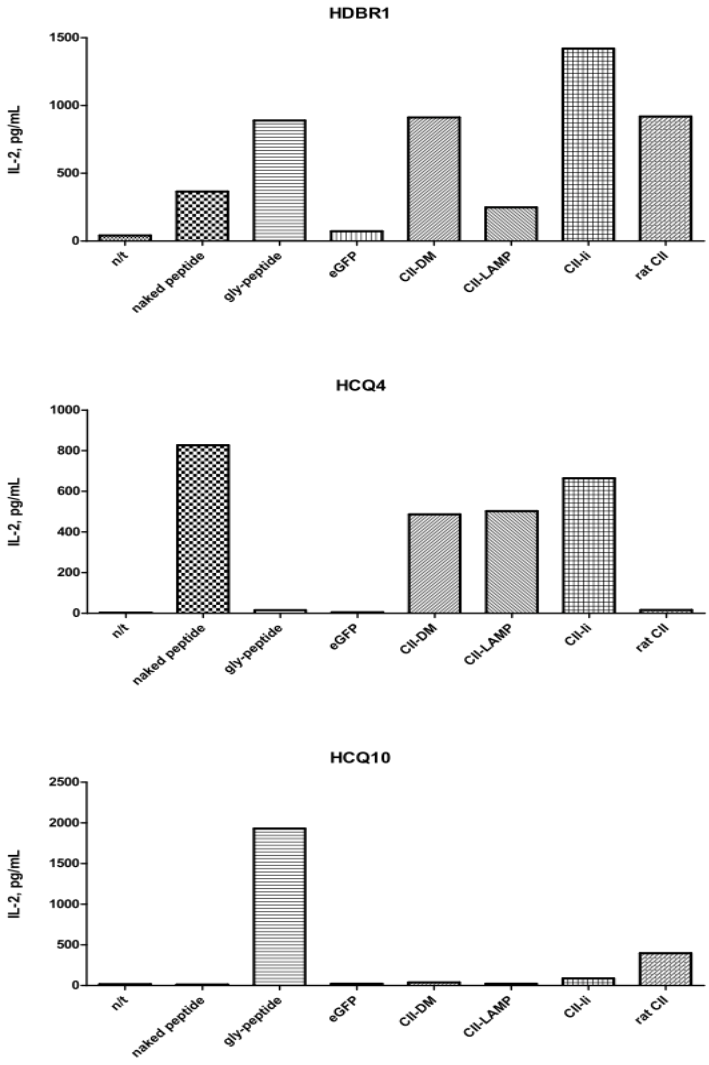


Figure 4.1 Stimulation of $CII_{259-273}$-responsive hybridomas by vCII-LAMP- and vCII-DM-transduced 3T3-I-A^q cells indicates $CII_{259-273}$-presentation.
IL-2 production measured by Sandwich ELISA and presented as sample concentration, in pg/mL in the 100-µL well, is taken as a marker of hybridoma response. HDBR1 responds most strongly to the CII-Ii-expressing 3T3-I-A^q cells but responds to 3T3-I-A^q cells treated with rat CII or the glycosylated $CII_{259-273}$ peptide more strongly than to 3T3-I-A^q cells treated with the naked $CII_{259-273}$ peptide, demonstrating a lack of specificity for one particular glycotype (both naked and glycosylated peptides kindly donated by Professor Holmdahl) . HCQ4 is strongly selective for the naked peptide and HCQ10 is selective for glycosylated isoforms. Hybridomas incubated with peptide aline (no 3T3-I-A^q) elicited no response (data not shown).

The experiment presented in figure 4.1 was repeated with the full set of hybridomas and with each well in triplicate, allowing hybridoma responses to be presented as given concentrations of IL-2 ± SEM. These data confirmed the initial findings that fusion protein expression in 3T3-I-Aq cells can drive $CII_{259-273}$-presentation to CD4+ hybridomas (figure 4.2).

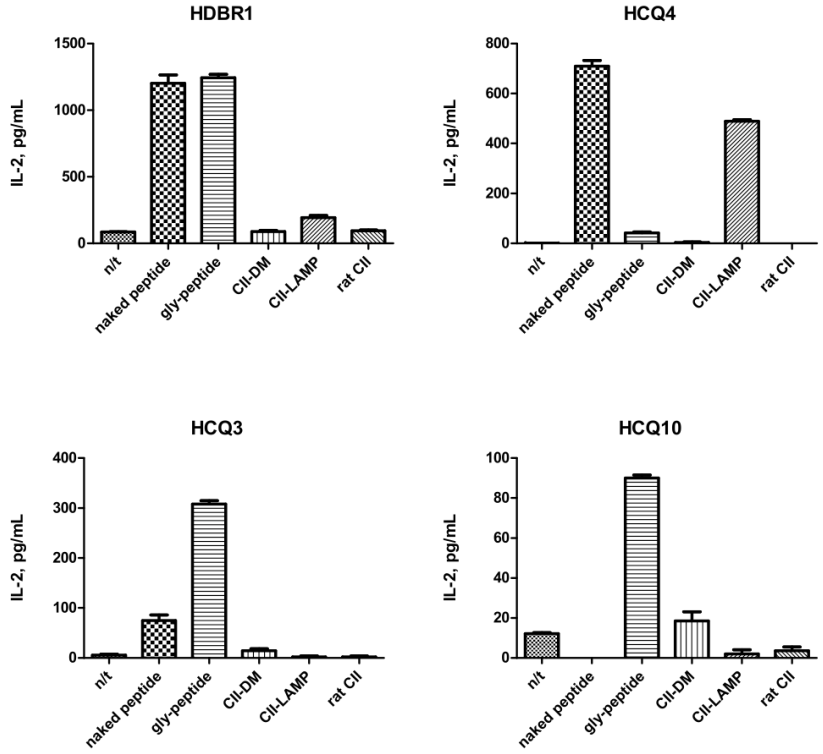


Figure 4.2 Similar response profile of CD4+ T cell hybridomas in second co-culture assay. HCQ3 and HCQ4 display a strong preference for 3T3-I-Aq cells treated with the glycolylated peptide or the naked peptide respectively. However, in contrast to the data presented in figure 4.1, CII-LAMP-expressing 3T3-I-Aq cells stimulate hybridoma HCQ4 more than CII-DM-expressing 3T3-I-Aq cells.

There are points of similarity and points of difference between these data presented in figures 4.1 and 4.2. Both HDBR1 and HCQ4 respond strongly to

stimulation with 3T3-I-A^q cells treated with the naked peptide whilst HCQ10 responds to 3T3-I-A^q cells treated with the glycosylated peptide. The overall magnitude of responses, measured by assaying the concentration of IL-2 in the culture media, did vary between experiments and an example of this is found in the readings obtained from hybridoma HCQ10, both in the data presented here and in other repeats of this experiment (data not shown). Whilst HCQ10 did always respond to the glycosylated peptide by producing IL-2 at levels far higher than in response to any other stimuli tested, the amount of IL-2 produced in response to 3T3-I-A^q cells treated with the glycosylated peptide varies wildly from experiment to experiment. This is because the overall responsiveness of HCQ10 was found to be temperamental in its overall ability to produce IL-2.

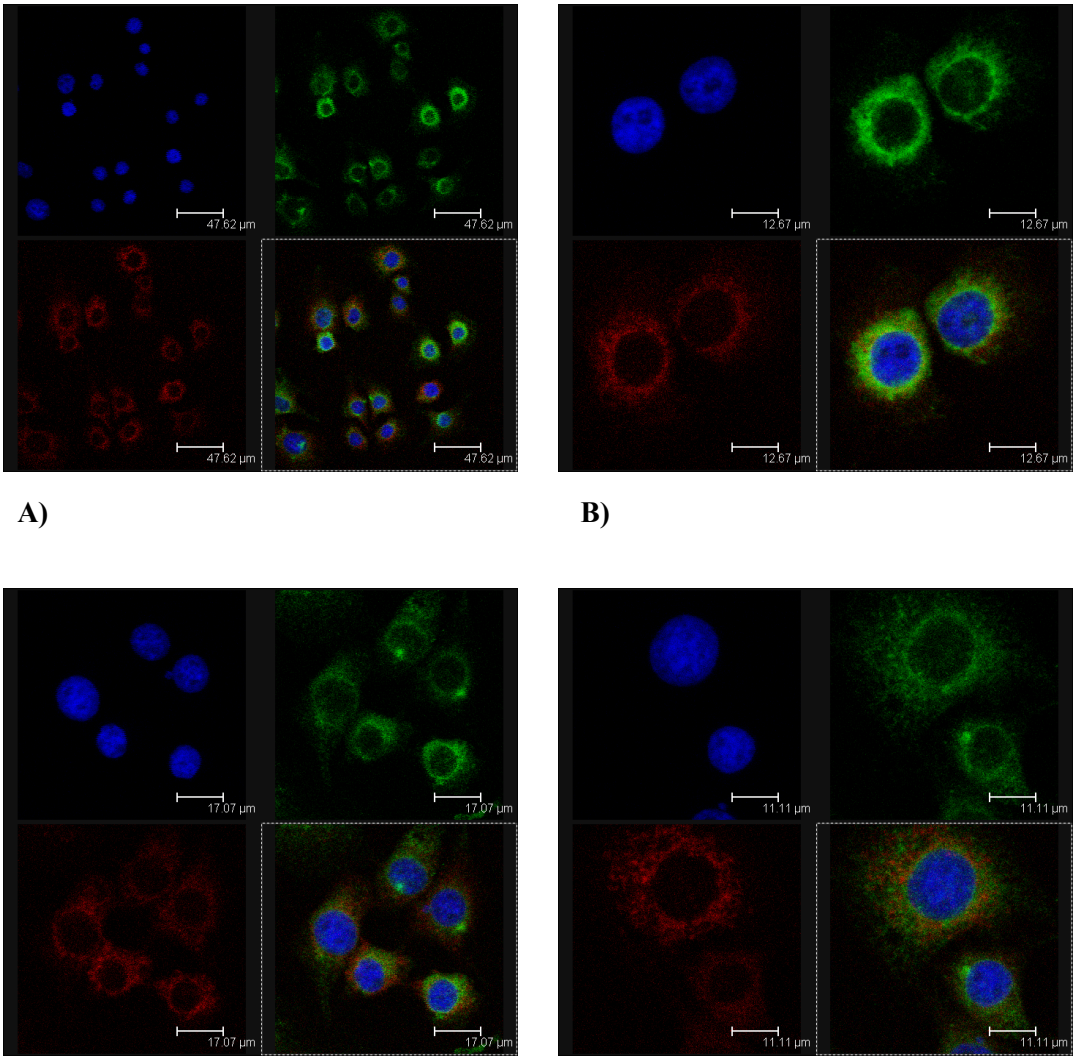
The temperamental nature of hybridoma responses is not confined to HCQ10. High levels of IL-2 are produced by these hybridomas only whilst they are expanding but although this is true of each line, HCQ10 were found to be particularly sensitive.

A second factor believed to give rise to variation between data from different experiments is the degree of confluence of 3T3-I-A^q cells, particularly in relation to their ability to process and present rat CII. Although care was taken to always seed the tissue culture plates with equal density of 3T3-I-A^q cells for each experiment, it was frequently noted that they failed to effectively process and present rat CII. Although frustrating, it is not altogether surprising as 3T3-I-A^q cells are an embryonic mouse fibroblast cell line, made to stably express MHC-I-A^q molecule and are not professional APCs. Nonetheless, these initial antigen presentation studies show that CII-LAMP is able to drive antigen presentation of the unglycosylated $CII_{259\text{-}273}$ peptide, inducing a response from HCQ4 hybridomas in the form of IL-2 production giving an excellent early indication that intracellular trafficking and proteolytic cleavage of the fusion protein occurs and that this is sufficient to result in $CII_{259\text{-}273}$ presentation.

Following investigation of the $CII_{259-273}$-presenting properties of vCII-LAMP- and vCII-DM-transduced 3T3-I-Aq cells, mouse BM-DCs were transduced and observed. Initial studies investigated the intracellular localisation of each fusion protein.

4.2.2 *Colocalisation studies of fusion proteins with intracellular markers in mBM- DCs*

Primary mouse dendritic cells were generated from total suspended bone marrow cell cultures with 20ng/ml rmGM-CSF as described in *Materials and methods*. After transduction with either vCII-LAMP or vCII-DM on day 4, cells were mounted on polylysine slides and fixed and permeablised on day 7. Initial studies aimed to compare the degree of colocalisation of fusion proteins with endogenous MHCII to see if either LAMP-1 or H2-DM-derived intracellular trafficking domain display better targeting to MHCII-containing compartments.



A) **B)**

C) **D)**

Figure 4.3 Confocal images of transduced primary mouse BM-DCs costained for MHCII. Cells were transduced with vCII-DM (a, b) or vCII-LAMP (c, d) and stained with DAPI nuclear stain (blue, first quadrant), and PE-conjugated anti-MHCII-I-A^q (red, third quadrant).

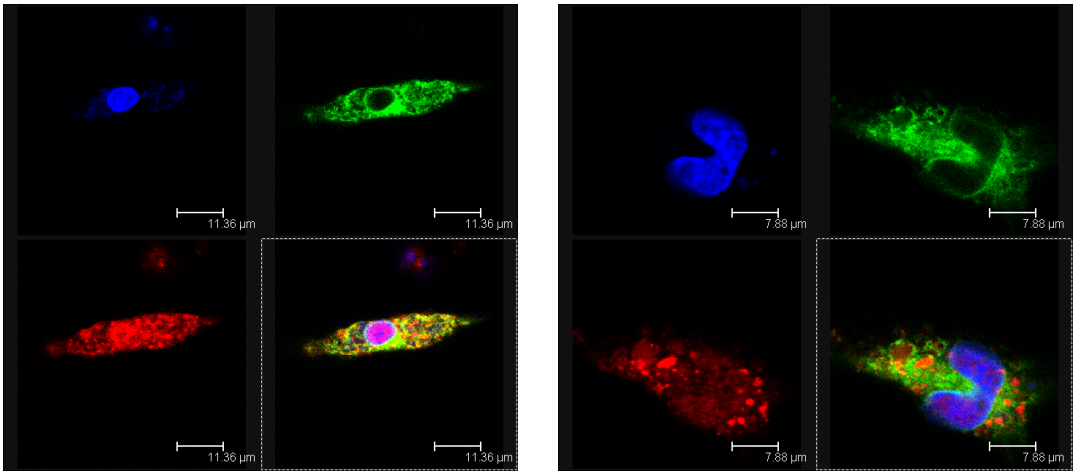
The data obtained by directly staining the cells with PE-labled anti-mouse MHCII-I-A^q shows faint but punctuate regions of MHCII-I-A^q density. Neither CII-LAMP nor CII-DM appeared to strongly co-localise with MHCII. Whilst strong colocalisation with MHCII might suggest that fusion protein-derived antigen loading

would be possible, the absence of strict colocalisation does not preclude this. It is known that a large proportion of MHCII in immature DCs recycles from the plasma membrane (Santambrogio & Strominger 2006) and it is conceivable that this accounts for the MHCII that does not colocalise with the eGFP-tagged fusion proteins. It was therefore decided to investigate the degree of colocalisation between fusion proteins and other components of the endocytic pathway

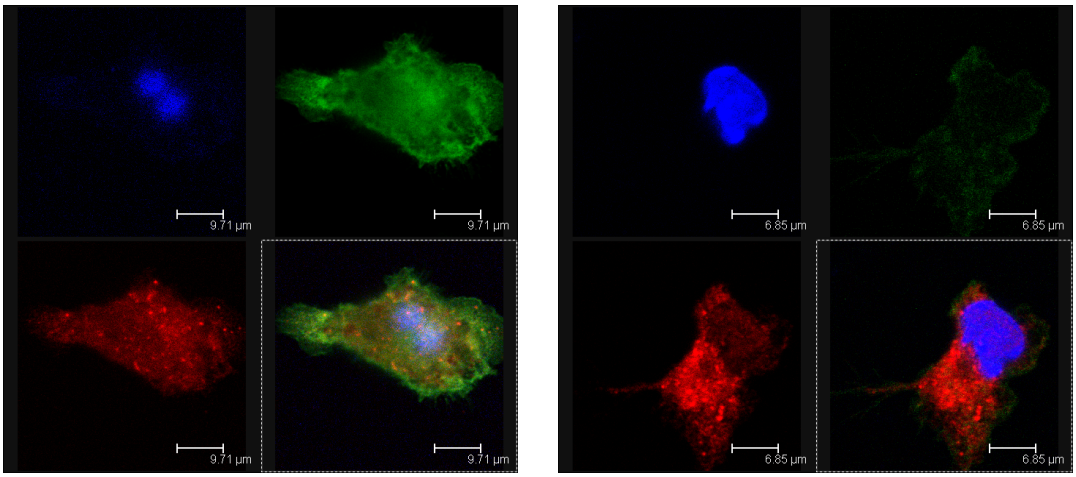
Fluorochrome-conjugated secondary antibodies were used in colocalisation studies of fluorescent-green fusion proteins with both Early endosomal antigen 1 (EEA-1) and LAMP-1 to enhance the signal of both the fusion protein and the intracellular marker. Biotinylated Goat anti-eGFP primary antibody followed by streptavidin-FITC secondary was used to increase eGFP signal (second quadrants, figures 4.4 and 4.5) and to ensure that even partially degraded or pH-quenched fluorescent proteins are detectable after fixing and permeablisation. EEA-1 was visualised using rabbit polyclonal anti-EEA-1 followed by Cy5-conjugated goat anti-rabbit (third quadrant, figure 4.4) whilst LAMP-1 was visualised by using rat monoclonal anti-LAMP-1 primary stain followed by Cy5-conjugated goat anti-rat (third quadrant, figure 4.5).

4.2.3 *Comparison of endosome and lysosome colocalisation of CII-LAMP and CII-DM*

EEA-1 is an early-endosomal membrane protein essential for fusion between early endocytic vesicles. Co-localisation with fluorescent proteins would indicate that a proportion of the fusion proteins are being trafficked through the early endosomal compartments. CII-DM was observed co-localising with EEA-1 to a large degree whereas CII-LAMP does not appear to display strong colocalisation with EEA-1 (figure 4.4 A and B). In contrast, regions of high CII-DM density were not always found to colocalise with endogenous LAMP-1 (figure 4.5 C), whilst CII-LAMP was usually found to colocalise strongly with endogenous LAMP-1 (figure 4.5 A and B).

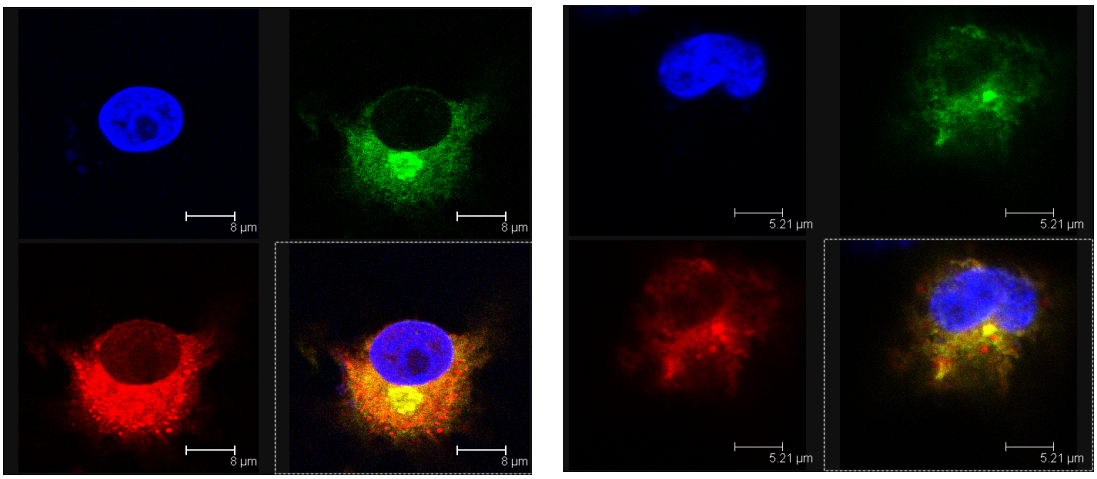


A) **B)**

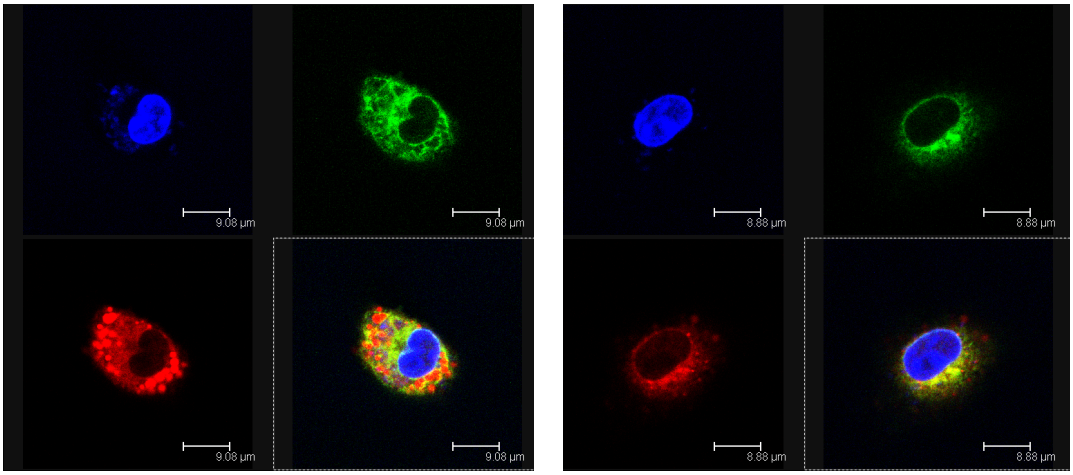


C) **D)**

Figure 4.4 Confocal images of primary mouse BM-DCs costained for endosomal marker EEA-1. Cells were transduced with vCII-LAMP (A), vCII-DM (B), veGFP (C), or untransduced (D) and stained with DAPI nuclear stain (blue, first quadrant), rabbit poly-clonal anti-EEA-1 followed by Cy5-conjugated goat anti-rabbit (red, third quadrant). EGFP signal was enhanced by staining with biotinylated goat anti-eGFP followed by streptavidin-FITC. All images are single-slice cross-sectional images. (Illumination of untransduced cells with the 488 laser, used to exite eGFP or FITC, did not result in a signal above background fluorescence, D, third quadrant).



A) **B)**



C) **D)**

Figure 4.5 Confocal images of transduced primary mouse BM-DCs costained for lysosomal marker LAMP-1. Cells were transduced with vCII-LAMP (A, B), vCII-DM (C, D) and stained with DAPI nuclear stain (blue, first quadrant), rat monoclonal anti-LAMP-1 followed by Cy5-conjugated goat anti-rat (red, third quadrant). EGFP signal was enhanced by staining with biotinylated goat anti-eGFP followed by streptavidinated-FITC.

Figures 4.4 and 4.5 suggest a difference in subcellular location of CII-LAMP and CII-DM fusion proteins in mouse BM-DCs namely that fusion protein CII-

LAMP localises to the lysosome more strongly than CII-DM. The aim of this project was to use *lvv* to induce specific tolerance to the $C_{259-273}$ peptide via presentation by APCs. Before taking either vector forward for use in a mouse study of tolerance induction in CIA, the efficacy of each *lvv* in driving $C_{259-273}$-presentation in primary mouse APCs was compared, again using BM-DCs.

4.2.4 *Mouse BM-DCs transduced with vCII-LAMP present $CII_{259-273}$ to CD4+ T cell hybridomas more efficiently than BM-DCs transduced with vCII-DM*

Experiments that co-cultured mBM-DCs with CD4+ hybridomas were performed to compare levels of $CII_{259-273}$-presentation by BM-DCs treated with different *lvv* and peptides. Due to the difficulty in sychronising the growth of several hybridoma cell lines, the added time constraint of using primary cells, and the fact that HCQ3 and HCQ4 are able to respond selectively to the glycosylated and naked $CII_{259-273}$ peptides respectively, these hybridomas alone were selected for co-culture.

Bone marrow extracted from the hind legs of DBA/1 mice was induced to differentiate down the DC lineage by culture in the presence of rmGM-CSF as described in *Materials and methods*. On day 3 100,000 cells were seeded into 24-well plates and either transduced at an MOI of 20 or treated with 100 μg rat CII. Addition of hybridomas HCQ3 and HCQ4 was performed on day 5 at the time of addition of fresh either naked or gly-peptide controls.

The first experiment was performed with or without addition of LPS one day after DC transduction/treatment with rat CII to determine whether induced maturation might enhance antigen presentation (fig 4.6).

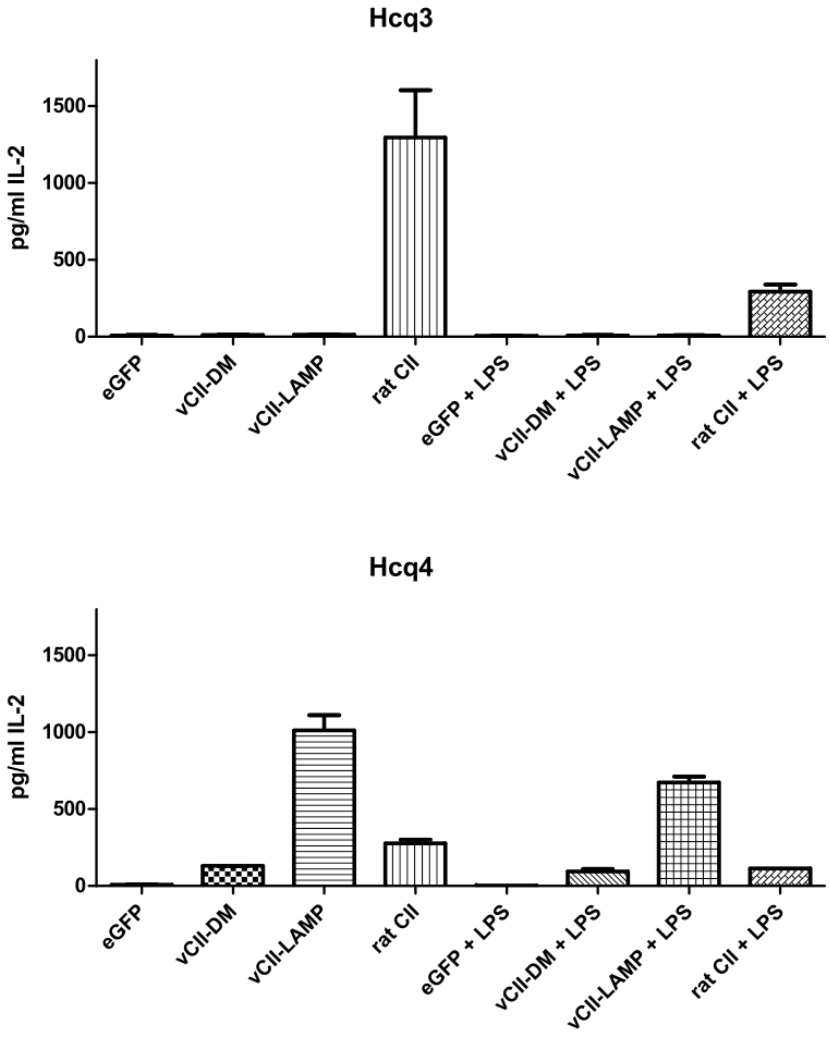


Figure 4.6 Antigen presentation by mBM-DCs to CD4+ T cell hybridomas with or without LPS. Both vCII-DM and vCII-LAMP-treated mBM-DCs induce a response from HCQ4 hybridomas, with vCII-LAMP inducing a response approximately five-fold stronger than vCII-DM, significantly higher ($p = 0.013$). Expression of neither CII-LAMP nor CII-DM in mBM-DCs induced stimulation of HCQ3 indicating an absence of glycosylation of the $CII_{259-273}$ epitope. Error bars represent SEM.

These data, obtained from primary mouse BM-DCs show similarities and differences from the data obtained from 3T3-I-A^q cells in figures 4.1 and 4.2. Clearly, these cells are able to efficiently take up and present rat CII more efficiently

than 3T3-I-A^q cells. This is not surprising given that DCs are professional APCs. It is clear from figure 4.5 that addition of LPS one day after transduction or rat collagen-treatment does not enhance, but reduces antigen presentation. Hence, LPS was not used in subsequent mBM-DC antigen presentation assays. Instead, the experiment was repeated using the naked $CII_{259-273}$ peptide and glycosylated $CII_{259-273}$ peptide controls (figure 4.7). Figure 4.5 suggests that mBM-DCs transduced with vCII-LAMP present the $CII_{259-273}$ peptide to HCQ4 more efficiently than vCII-DM-transduced cells. This was also seen in figure 4.6.

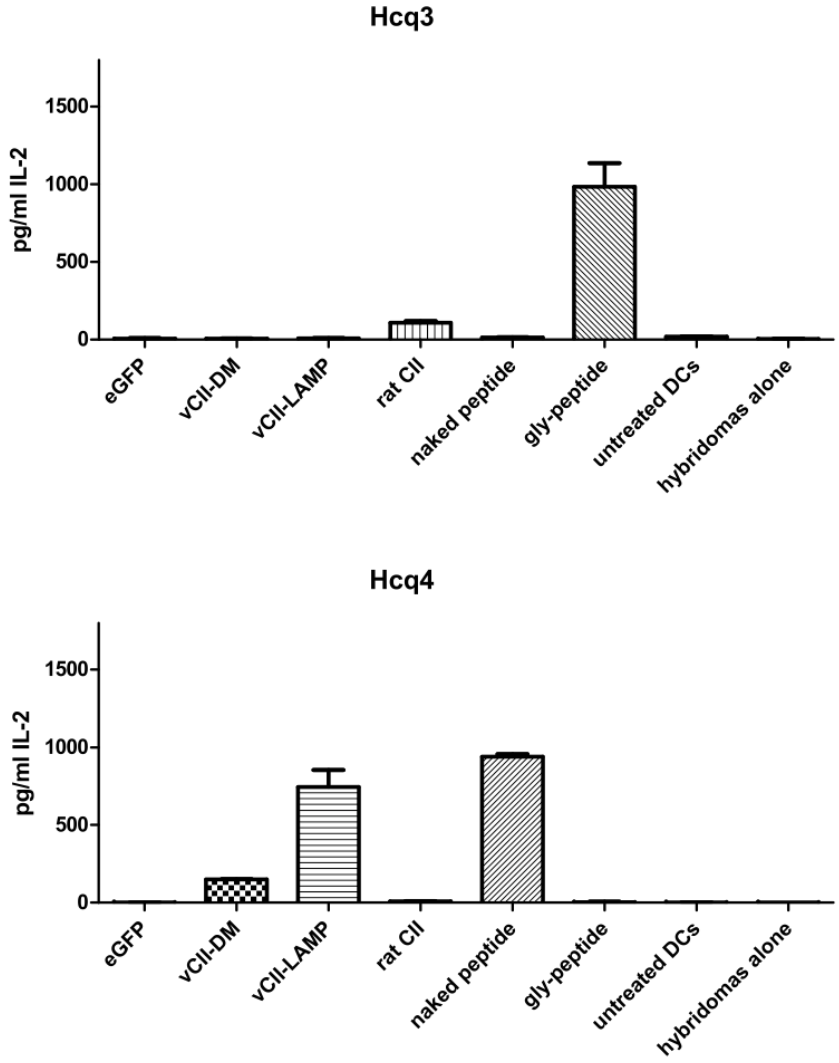


Figure 4.7 Antigen presentation by mBM-DCs to CD4+ T cell hybridomas. IL-2 production-response by HCQ3 is highly selective for the glycosylated peptide whilst HCQ4 responds instead to the naked peptide. vCII-LAMP induces a response four-fold greater than vCII-DM but both fusion proteins result only in presentation of the naked peptide. The difference in HCQ4 response to vCII-LAMP-treated mBM-DCs compared with the response to naked peptide-treated mBM-DCs is not considered significant ($p = 0.22$)

The results of the antigen-presentation assays clearly demonstrate that vCII-LAMP induces $CII_{259-273}$-presentation with higher efficiency than an equal infectious

dose of vCII-DM. Therefore vCII-LAMP was selected for use in animal experiments described in chapter 5 that determine the efficacy of endosome/lysosome targeted T cell epitopes for specific tolerance induction.

Whilst the experiments presented above have used eGFP-expressing *lvv* as a control and previous studies, in the case of Ii-based fusion proteins for intracellular targeting of the $CII_{259-273}$ epitope, used an Ii-expressing *lvv* control (Gjertsson et al. 2009) a more relevant control in this study would be a *lvv* that delivers a fusion construct identical to CII-LAMP in every way but for the peptide between the two cathepsin S cleavage sites. Whilst "empty vector" constructs, lacking a peptide between the cathepsin sites, and "scrambled collagen" constructs, containing a sequence that encodes the same amino acids as the $CII_{259-273}$ 15-mer but in a random order were cloned and viral vectors prepared, it was decided that the best control would deliver a peptide that also binds the MHCII I-Aq molecule but which is irrelevant to CIA. The MOG_{79-90} peptide binds I-Aq molecules and is sufficient to cause experimental autoimmune encephalitis (EAE) in DBA/1 mice in the absence of adjuvant (Abdul-Majid et al. 2000). A construct incorporating a sequence encoding the MOG_{79-90} peptide between the cathepsin S cleavage sites was generated, denoted pMOG-LAMP, and lenti-viral vectors to deliver this construct were prepared, denoted vMOG-LAMP.

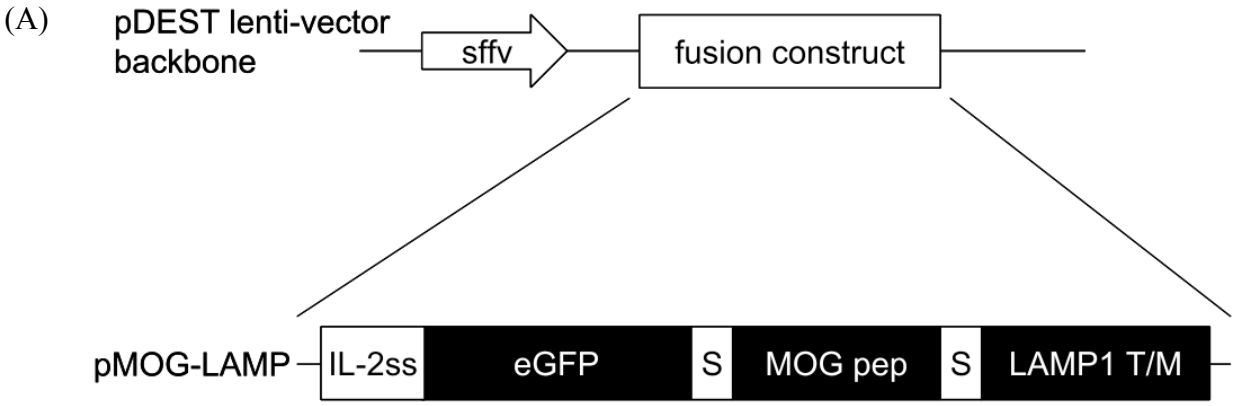
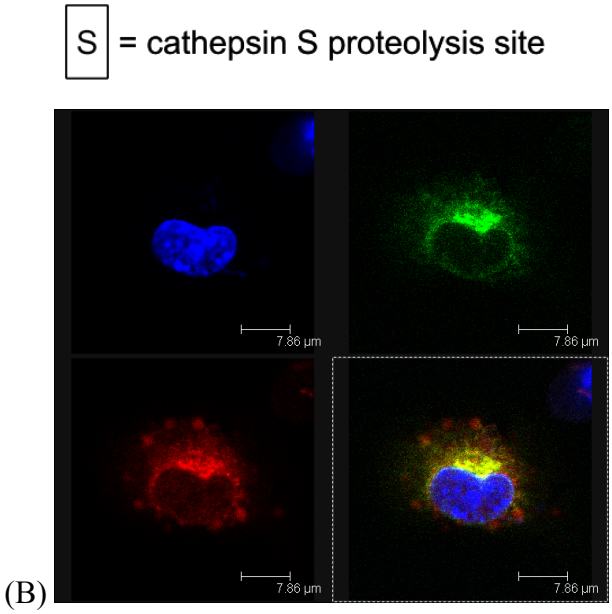


Figure 4.8 Schematic cartoon and micrograph of pMOG-LAMP. (A) DNA plasmid identical to pCII-LAMP except for the peptide region that encodes MOG_{79-90}. (B) Confocal micrograph at 1000-fold magnification shows the eGFP-tagged (green, second quadrant) pMOG-LAMP fusion protein colocalises with rat monoclonal anti-LAMP-1 followed by Cy5-conjugated goat anti-rat (red, third quadrant) strongly indicating that the full-length protein is being expressed. Blue, first quadrant: DAPI nuclear stain. Fourth quadrant: combined.



The aim of this study is to show that *lvv*-mediated MHCII-presentation of a peptide can drive specific tolerance to that peptide. Recognition by a CD4+ T cell of any MHCII-bound peptide in the absence of costimulation may direct the T cell to become an immunosuppressive Treg cell as outlined above. Treg cells exert their suppressive effects via multiple contact-dependent and contact-independent mechanisms some of which are not antigen-specific (La 2009). Hence, there is the possibility that *lvv*-driven MHCII/peptide-presentation might induce Treg

development that subsequently causes a non-specific immunosuppression reducing the severity of autoimmune conditions such as CIA. By using a control lentiviral construct that delivers a peptide that is not involved in CIA but is known to bind MHCII-I-A^q, such a global immunosuppression due to potential bystander effects can be excluded. The vMOG-LAMP vector was selected for the control group of mice to control for bystander suppression.

Unfortunately a MOG_{79-90}-responsive CD4+ T cell hybridoma was not available to assay MHCII-presentation of the MOG_{79-90} epitope. A recent report of the use of soluble MHCII-I-A^q bound to the MOG_{79-90} peptide was also unable to directly or indirectly demonstrate the presence of MOG_{79-90} bound to the MHCII molecule (Batsalova et al. 2010). After vMOG-LAMP was generated and titred, it was used, alongside vCII-LAMP, to transduce mBM-DCs which were then co-cultured with HCQ3 and HCQ4 T cell hybridomas to ensure it was unable to cause stimulation of $CII_{259-273}$-reactive T cells (figure 4.8).

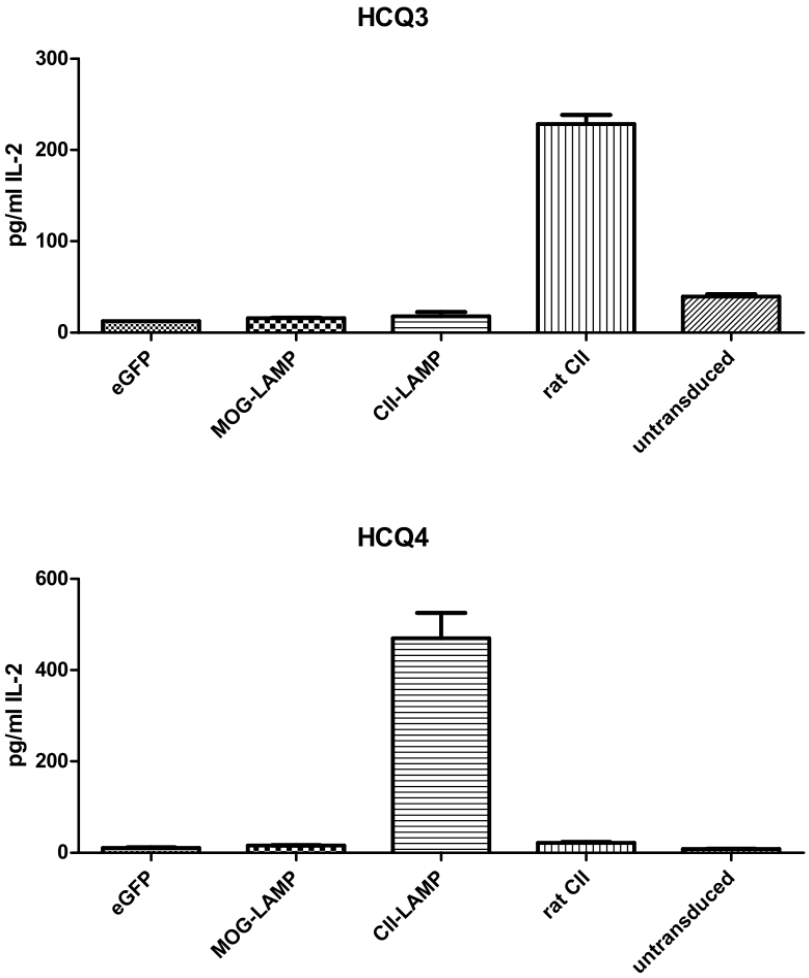


Figure 4.9 MOG-LAMP-expressing mBM-DCs do not stimulate CD4+ T cell hybridomas HCQ3 and HCQ4. Neither HCQ3 nor HCQ4 hybridoma responded to co-culture with vMOG-LAMP-treated mBM-DCs.

4.3.1 *Summary*

The aim of the work presented in this chapter was primarily to assess the ability of the novel *lvv*'s vCII-LAMP and vCII-DM, described in chapter 3, to induce surface presentation of the $CII_{259-273}$ epitope. Also important was the need to compare the two fusion proteins' ability to drive antigen presentation. These tests were initially performed in the mouse fibroblast cell line 3T3-I-A^q.

These first analyses of *lvv*-driven antigen presentation utilised all four available CD4+ T cell hybridomas, reportedly responsive to distinct glycosylation states of the $CII_{259-273}$ peptide. Figures 4.1 and 4.2, however, do not completely agree with the reported glycotype specificities as summarised in table 4.1. In particular HDBR1 was found to strongly react to the glycosylated peptide that carries a galactosylated-hydroxylysine at position 264 (the consensus glycotype in native collagen), contrary to its reported selectivity for hydroxylysine only. Based on these initial data, HCQ3 and HCQ4 were selected as sole responder cells for use with primary mouse APCs as these hybridomas alone give a reliable indication as to the relative efficiency of naked $CII_{259-273}$ peptide presentation verses glycosylated $CII_{259-273}$ peptide presentation. These hybridoma lines have been used alone elsewhere to determine the glycosylation state of $CII_{259-273}$ where they were used to show that the administration with glycosylated $CII_{259-273}$ peptide in complex with solublised MHC-I-A^q molecules prevented development of CIA in mice and ameliorated chronic relapsing disease whereas the naked $CII_{259-273}$ peptide in complex with solubilised MHC-I-A^q molecules had no such effect (Dzhambazov et al. 2006).

Primary mBM-DCs transduced with vCII-LAMP and vCII-DM stimulate $CII_{259-273}$-specific T cell hybridomas to produce IL-2 providing direct evidence that expression of CII-LAMP or CII-DM in mouse APCs results in processing of the fusion protein and surface presentation of the peptide on MHCII-I-A^q molecules. Confocal micrographs revealed a stronger localisation of CII-LAMP to lysosome-like compartments and IL-2 production by HCQ4 in response to vCII-LAMP-treated

mBM-DCs was repeatedly shown to be 4 to 5 fold higher than in response to mBM-DCs treated with equivalent infectious load of vCII-DM. Hence, vCII-LAMP was selected for the *in vivo* studies presented in chapter 5.

Before vCII-LAMP was tested in mice, the vMOG-LAMP control was designed to deliver the MOG_{79-90} peptide, irrelevant in autoimmune arthritis but known to bind the MHCII-I-A^q molecule. The vMOG-LAMP *lvv* was prepared and tested alongside vCII-LAMP in an antigen-presentation assay to CII-responsive hybridomas. As expected, vMOG-LAMP did not elicit an IL-2 secretion response from either hybridoma line.

4.3.2 *Discussion*

One potential criticism of the antigen-presentation data presented in this chapter is that the absolute values of IL-2 produced, by particular hybridomas and, in some cases, to particular antigen-presentation stimuli, were not always consistent between different experiments. It is argued here that, due to the nature of the experimental systems used, much of this variation was unavoidable. Further, it is believed that, despite variability of IL-2-production in response to some antigen-presentation stimuli, the data obtained provides a strong enough indication that vCII-LAMP is a *lvv* capable of inducing highly detectable levels of surface $CII_{259-273}$ peptide presentation to take this *lvv* forward for testing *iv vivo*.

The overall, maximal level of IL-2 production of a given hybridoma line was subject to variation between experiments. For maximum IL-2 production to be achieved, these hybridomas need to be co-cultured with APCs during rapid expansion, a phase of hybridoma growth that begins 2-5 days after thawing and lasts for 2-4 days depending on the hybridoma line. As noted previously, this made the timing of hybridoma thaw critical for obtaining data. Unfortunately, sometimes, as in the case of HCQ10 in figure 4.2, one hybridoma line was not undergoing rapid expansion at the time of co-culture and this resulted in the level of IL-2 production from the hybridoma varying by as much as 15-fold from experiment to experiment.

The timing of addition of rat CII to 3T3-I-A^q cells was similarly delicate with good processing and presentation of rat CII by these cells unreliable. What appears as a strong response from hybridomas HBDR1 and HCQ10 to rat CII presented by 3T3-I-A^q cells in figure 4.1 was not repeated in figure 4.2. As noted in section 4.2.1 this unreliability of collagen processing and MHC class II-presentation is not surprising in cells that are not professional APCs.

Despite these limitations, overall, the data presented in this chapter, and in particular the antigen presentation data obtained from the primary mBM-DCs strongly indicate that the fusion proteins CII-LAMP and CII-DM are processed giving rise to strongly detectable surface MHC-I-A^q-$CII_{259-273}$ peptide presentation with *lvv* vCII-LAMP inducing the stronger responses from HCQ4 hybridomas co-cultured with transduced mBM-DCs.

Chapter 5. Results III

Vaccination with vCII-LAMP is protective in CIA

5 Vaccination with vCII-LAMP is protective in CIA

5.1 *Introduction*

Efficient MHCII-presentation of the $CII_{259-273}$ epitope by primary, immature mBM-DCs transduced with vCII-LAMP was shown and discussed in chapter 4. It was hypothesised that, if this virus could similarly drive MHCII-presentation of $CII_{259-273}$ *in vivo*, this might induce specific tolerance to CII. The control virus used was vMOG-LAMP, also discussed in the previous chapter. This chapter presents the results of the *in vivo* experiments which show that vaccinantion with vCII-LAMP confers protection from CIA. Biological data were sought to elucidate the underlying mechanism of this protection. These data are also presented here.

5.2 *Significant amelioration of CIA via tail vein injection of vCII-LAMP*

The experimental protocols used to test vCII-LAMP in the CIA mouse model of autoimmune arthritis closely followed those employed by the Ii-based $CII_{259-273}$-delivery study (Gjertsson et al. 2009) and were performed in collaboration with this group. In brief, adult male mice were vaccinated with 5 x 10^6 infectious units of either vCII-LAMP or vMOG-LAMP by tail vein injection. 28 days later, denoted day 0, the first rat CII injection was administered intradermally in Freund's complete adjuvant with a booster (CII in Freund's incomplete adjuvant) being administered on day 21 *ip*. Arthritic disease was scored by assessing the degree of swelling or erythema in joints and digits; an inflamed joint being scored 1-3 depending on severity and each inflamed digit receiving a score of 0.25.

Groups of 10 mice were kept in a cage under standard conditions, one group being administered each with 5 x 10^6 infectious units of vCII-LAMP and the second group with 5 x 10^6 infectious units of vMOG-LAMP per mouse. The experiment was performed twice using the same reagents at the same time points. Arthritic score was also measured on the same days such that the data from each experiment could be compiled (figure 5.1). Termination of each experiment, the first after 39 days and the

second after 42 days, immediately proceeded the first day that the value for the average arthritic score of the vCII-LAMP-treated mice increased by approximately one arthritic score unit.

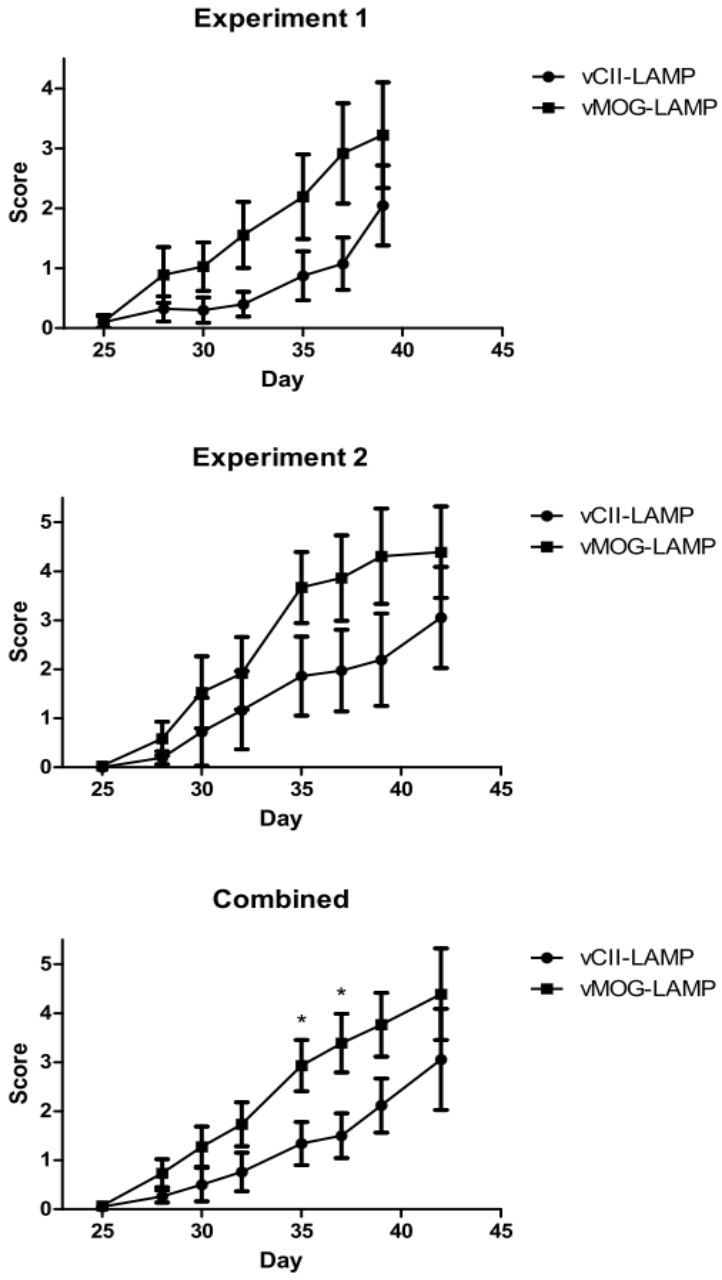


Figure 5.1 Mean arthritic score from experiment 1, experiment 2 and combined. 18 Mice were used in experiment 1, nine in each group while 19 mice were used in experiment 2, ten receiving vCII-LAMP and nine receiving the vMOG-LAMP control virus. All mice were vaccinated with 5 x 10^6

infectious units of recombinant lvv 28 days prior to CII/CFA administration. CII/IFA was administered on day 21. The data from each experiment was collected on equivalent days and compiled to give the *combined* plot. Mann-Whitney U test of time-points 35 and 37 have asociated p values of 0.0487 and 0.0440 respectively. All other differences between the groups have associated p values of above 0.05.

Whilst both experiments appear to clearly show the protective effect of vaccination with vCII-LAMP, only when data from both experiments are combined do the differences, as measured by performing the 2-tailed Mann-Whitney U test, become significant (with p values below 0.05). The time points at which the p values fall below this threshold were on days 35 and 37. Thus, together, these experiments demonstrate that *lvv* that express the novel fusion protein CII-LAMP provide DBA/1 mice with significant protection from CIA at these time points.

Although severity of disease was largely reduced, there was no overall reduction in disease incidence. In the first experiment, three vCII-LAMP-treated mice still showed no clinical signs of inflammation by the end of the experiment compared to two of the mice treated with vMOG-LAMP. The second experiment ended with one vMOG-LAMP-treated mouse unaffected with all mice in the vCII-LAMP group showing some sign of inflammation, although two of these mice only had a clinical score of 0.25 meaning a single digit showed some redness.

The scale of measurement of arthritic score is not a true continuum as the values are averages of scores attributed to individual mice that can vary only by increments of 0.25 or more. Furthermore, scores of 0.25 per inflamed digit and 1-3 per inflamed joint are arbitrary ranking values, hence this is an ordinal scale meaning the data is non-parametric. Therefore, the statistical analysis of variance appropriate for these data is a Mann Whitney U test.

It is deduced that the tolerisation induced in these mice is due to the $CII_{259-273}$ peptide that forms a part of the fusion protein expressed by vCII-LAMP, the only element that differs in the construct expressed by the otherwise identical *lvv* vMOG-

LAMP. The mechanism by which expression of CII-LAMP affects tolerisation was investigated through analysis of biological data collected from blood samples at day 28 and upon termination of each experiment.

5.3 *Lymphoproliferation in response to rat CII*

A direct measurement of the degree of immunity an experimental animal has to an antigen is made by exposing splenocytes or lymph node cells to the antigen and measuring the proliferative response. The spleens and draining lymph nodes were harvested from five mice from each group upon termination of experiment 1. Single-cell cultures were stimulated with either 50 μg/mL rat CII or 1.25 μg/mL of Con A, the sugar-binding lectin and lymphocyte mitogen (Mackler 1972) as a positive control. Con A is a useful positive control as it a powerful lymphocyte mitogen at concentrations in the order of 1.25 μg/mL as used here. After 72 hours 1 μCi [^{3}H]-thymidine was added and 20 hours later cells were washed and measured for [^{3}H]-thymidine uptake, a measure of growth-rate. Differences in the proliferative response, as measured in counts per minute (CPM), between cells from the two different groups of mice stimulated with the same stimuli were not significant, although there were some trends; both unstimulated cells from vCII-LAMP-treated mice and cells from vCII-treated mice stimulated with rat CII displayed lower levels of [^{3}H]-thymidine incorporation than cells from the vMOG-LAMP-treated group exposed to the same stimuli. There was no such trend between cells from each group of mice that were stimulated with Con A as a positive control for [^{3}H]-thymidine uptake (figure 5.2).

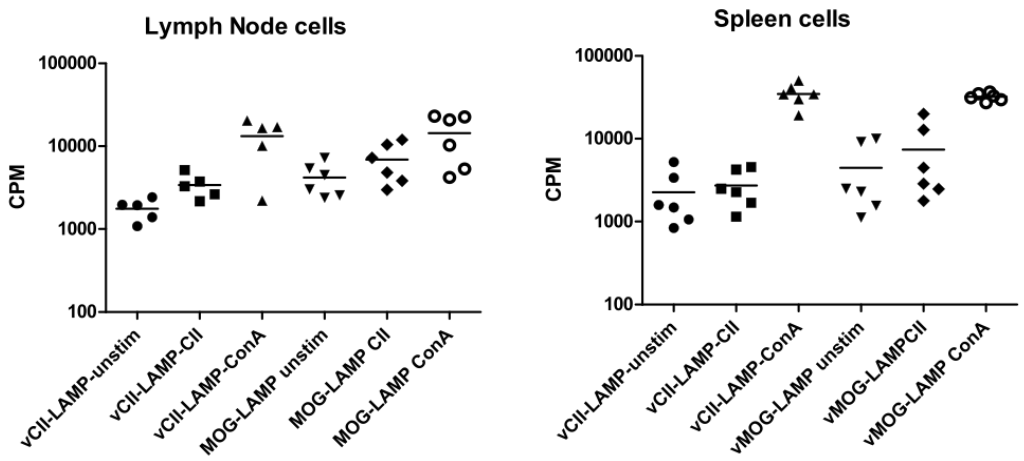


Figure 5.2 Cell proliferation in response to antigen or mitogen. 2 x 10^6 cells were stimulated with rat CII, Con A or no stimulation for 72 hours and a further 20 hours in the presence of tritiated thymidine. Cell growth is quantified by measuring the uptake of radiolabeled thymidine in counts per minute (CPM). No significant differences were observed between proliferation of cells from vCII-LAMP-treated mice and vMOG-LAMP-treated mice exposed to the same stimulus.

Although below the accepted significance threshold, analysing the variance between the data sets 'vCII-LAMP CII' and 'vMOG-LAMP CII' obtained from lymph node cells by performing a 2-tailed Student's t test give a p value of 0.074, indicating a strong trend. A trend of stronger proliferative responses was observed both in splenocytes and lymph node cells exposed to rat CII from vMOG-LAMP-treated mice than cells from vMOG-LAMP-treated mice.

5.4 *Cytokine assays*

Supernatants from lymph node cells that had been incubated with rat CII, Con A or left unstimulated were harvested after 72 hours for measurement of cytokine levels. Titres of IL-10, IL-17 and TGF-β, cytokines highly relevant in tolerance and autoimmunity, were measured using sandwich ELISA. TGF-β was not

present at levels detectable by the kits (above 20 pg/mL) whilst IL-10 and IL-17 was only detectable in those supernatants harvested from samples stimulated with Con A (figure 5.3). Splenocytes were also tested for TGF-β, IL-10 and IL-17 but in each case, the cytokine levels were lower than that for lymph node cells; all data points for IL-10 and IL-17 production in response to Con A were lower than 100 pg/mL and all other other points indistinguishable from background (data not shown).

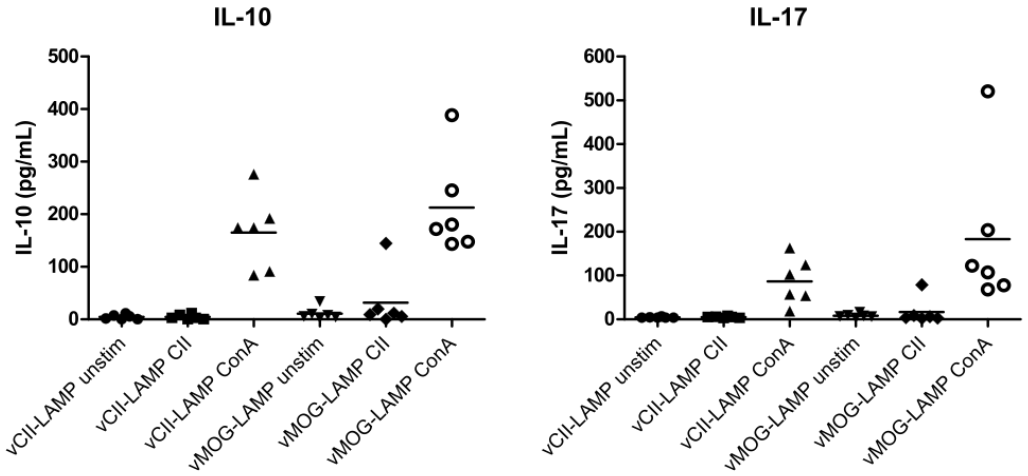


Figure 5.3 ELISA of IL-10 and IL-17 produced by stimulated draining lymph node cells. Student's t test analysis of the differences in IL-10 or IL-17 levels in the supernatants from draining lymph node cells from vCII-LAMP and vMOG-LAMP-treated mice stimulated with the same antigen/mitogen reveals no significant differences between the groups (ie. all such p values greater than 0.05).

Although many cytokines are involved in both CIA and RA, many are produced within the synovia of inflamed joints (Brennan & McInnes 2008) and were thus beyond the measure of this study. Other cytokines produced by re-stimulated lymph node cells or splenocytes that have been measured in previous studies include IFN-γ, IL-4, IL-6 and IL-12 (Gjertsson et al. 2009). As the supernatants from lymph node cell and spleen cell restimulation were only available for the cytokine assay by ELISA towards the very end of the present study after time

and funds had technically expired, it was necessary to be very selective about which cytokines to measure.

The previous study by Gjertsson *et al* found no differences in IFN-γ and IL-12 levels between the groups. There was also no difference in IL-17 levels between the groups in the Gjertsson *et al* study. However, as a crucial cytokine in autoimmunity it was decided to measure IL-17 levels to check that no differences were present in this case.

IL-4 was not found at detectable levels in the Gjertsson *et al* study and was not selected here.

IL-6 is not constitutively expressed but is readily induced by multiple stimuli and is found in sera of both RA patients and CIA mice (Park & Pillinger 2007). It could form a part of future research into the role of cytokine signaling in FP-*lvv*-induced antigen specific tolerance but was not amongst the three cytokines tested here.

5.5 *ELISA measurements of mouse serum antibody titre*

Sera from tail-vein bleeds were harvested at day 28 of experiment 1 and upon termination of both experiments. Samples were serially diluted in PBS and incubated on plates coated with either rat CII or a mouse-Ab-specific capture antibody. The amount of sera-derived Ab was then determined by using HRP-conjugated detection antibodies (Sandwich ELISA) followed by blue-colour substrate. No CII-binding standards were available to allow determination of absolute concentration of CII-specific Ab serotypes, so only comparative measurement between samples collected from vCII-LAMP-treated mice with vMOG-LAMP-treated mice was possible. Therefore, the raw $OD_{540\text{-}450}$ data are plotted. Because dilution factor and colour-reaction time both strongly affect the final $OD_{540\text{-}450}$ and because the correspondence between concentration and $OD_{540\text{-}450}$ is only considered linear up to $OD_{540\text{-}450}$ values of approximately unity, care was taken to terminate the reactions well before saturation occurs such that the readings

still fall in or around the linear range, thus maximising the resolution of differences between the groups. A comparison of high and low concentrations of the same samples is presented in figures 5.4A and 5.4B to illustrate this.

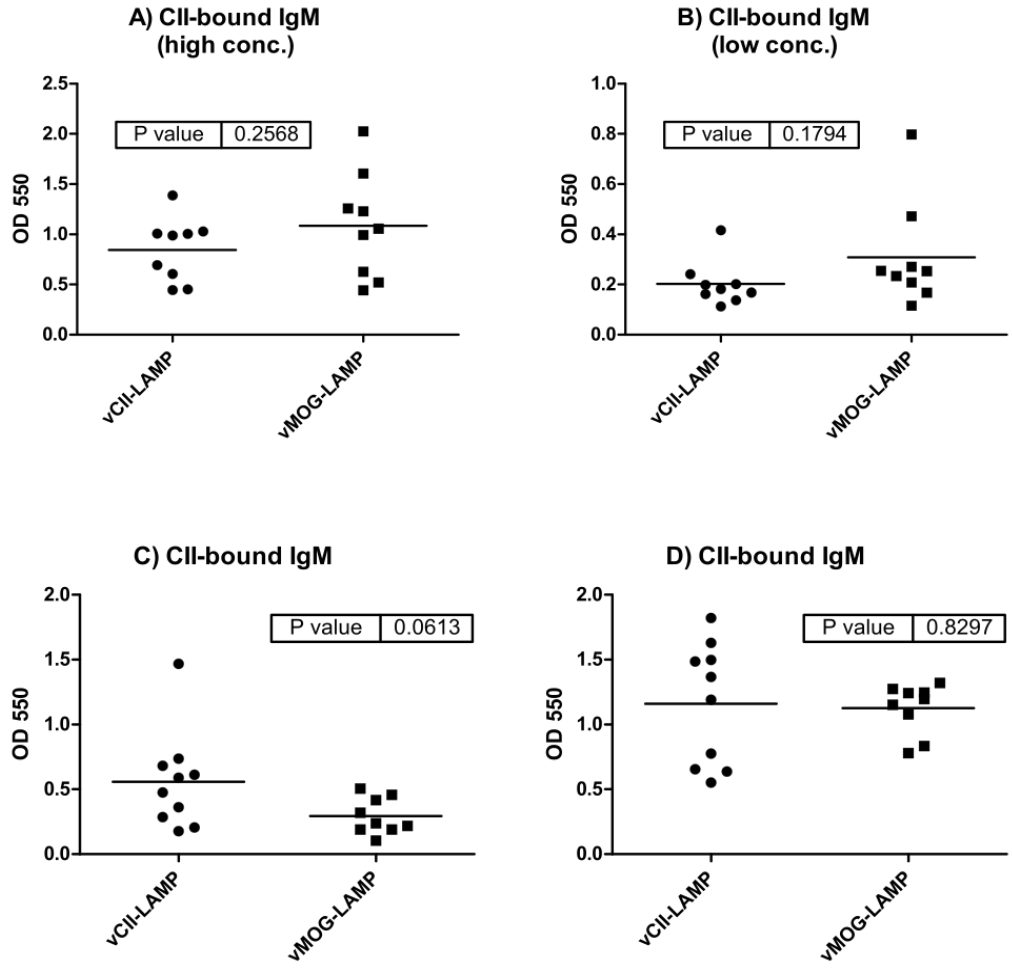


Figure 5.4 ELISA of CII-bound IgM. A and B) High and low dilutions of the same samples taken at the end point of experiment 2; C) Samples collected at day 28, experiment 1; D) Samples collected at the endpoint of experiment 1. P values are calculated using the two-tailed Student's t test assuming equal variance.

Figures 5.4A and 5.4B illustrate how a further dilution of the same samples (figure 5.4B) reduces the $OD_{540\text{-}450}$ values resolving differences between the groups,

thus lowering the p value. Figure 5.4C shows that in experiment 1, mice vaccinated with vCII-LAMP tended to have higher levels of CII-specific IgM on day 28, but this difference is not observed when the experiment is terminated on day 39 (figure 5.4D).

Figure 5.5 compares CII-specific IgG levels from the sera of vCII-LAMP and vMOG-LAMP-treated mice on day 28 from experiment 1 (5.5A), and upon termination of both experiments (5.5B and C)

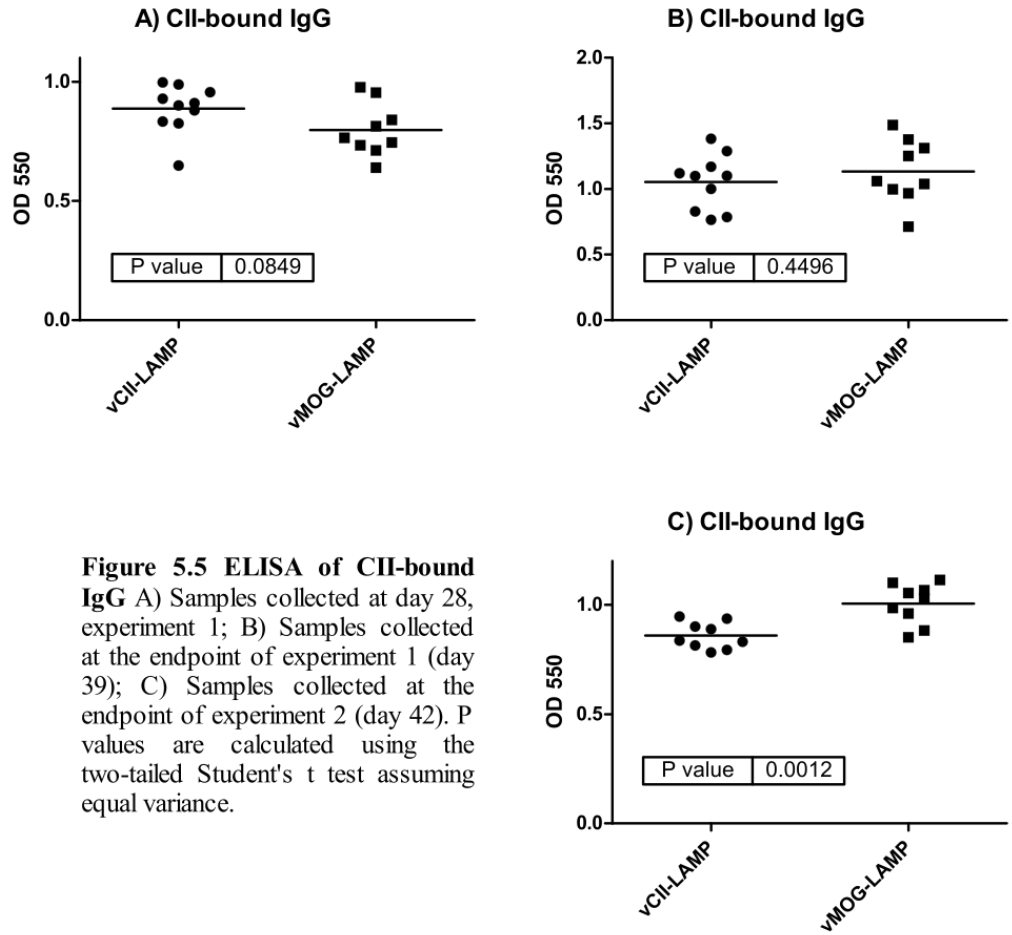


Figure 5.5 ELISA of CII-bound IgG A) Samples collected at day 28, experiment 1; B) Samples collected at the endpoint of experiment 1 (day 39); C) Samples collected at the endpoint of experiment 2 (day 42). P values are calculated using the two-tailed Student's t test assuming equal variance.

At the timepoint at which experiment 2 was terminated, day 42, mice vaccinated with vCII-LAMP have significantly lower CII-specific IgG titre. Because the IgG2A and IgG2B serotypes rather than IgG1 are able to bind complement and

have a strong correlation to arthritic disease, the titres of these serotypes were measured separately.

In figure 5.6, a pattern of CII-specific IgG2A levels similar to the total CII-specific IgG presented in figure 5.5, are seen. Again, titres in vCII-LAMP-treated mice are slightly higher at the mid-experiment time point (day 28, figure 5.6A), but this trend is reversed by day 39 of experiment 1 (figure 5.6B) The titre is significantly higher in samples from vMOG-LAMP-treated mice at the end-point of the second experiment (figure 5.6C). This pattern is again repeated in the IgG2B measurements (figure 5.7) although the differences are not significant.

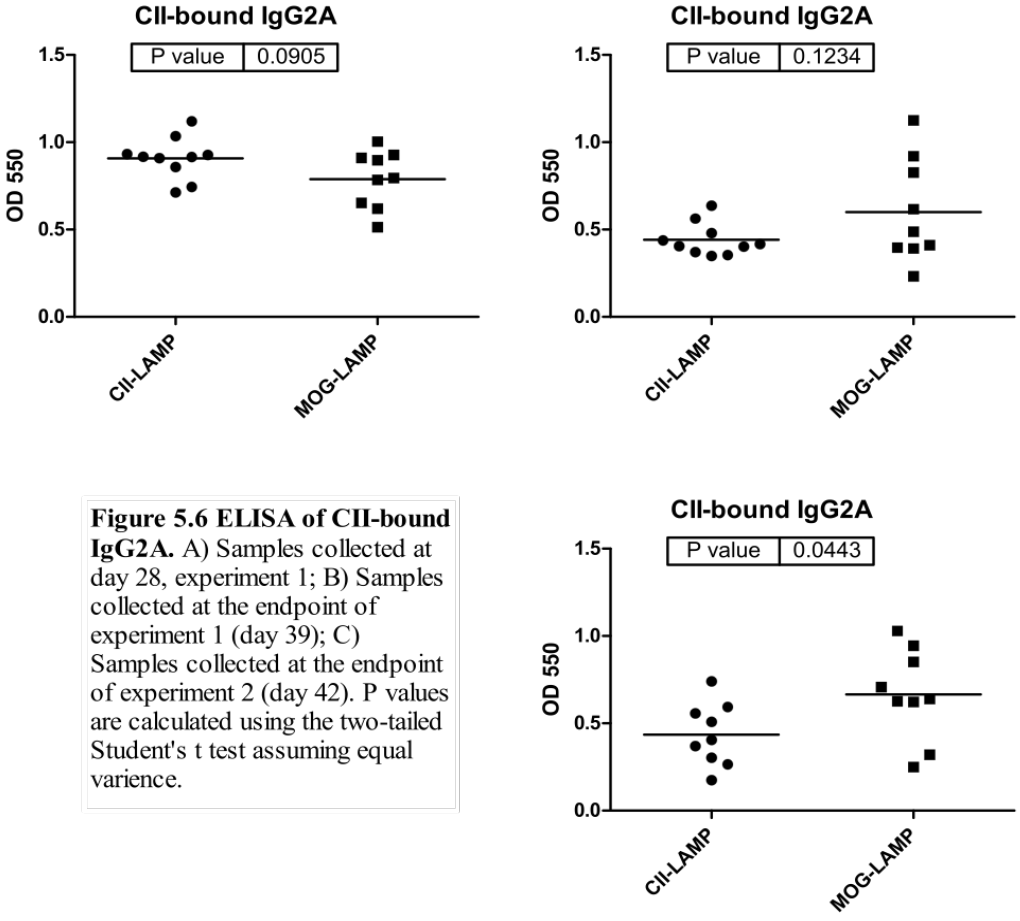


Figure 5.6 ELISA of CII-bound IgG2A. A) Samples collected at day 28, experiment 1; B) Samples collected at the endpoint of experiment 1 (day 39); C) Samples collected at the endpoint of experiment 2 (day 42). P values are calculated using the two-tailed Student's t test assuming equal varience.

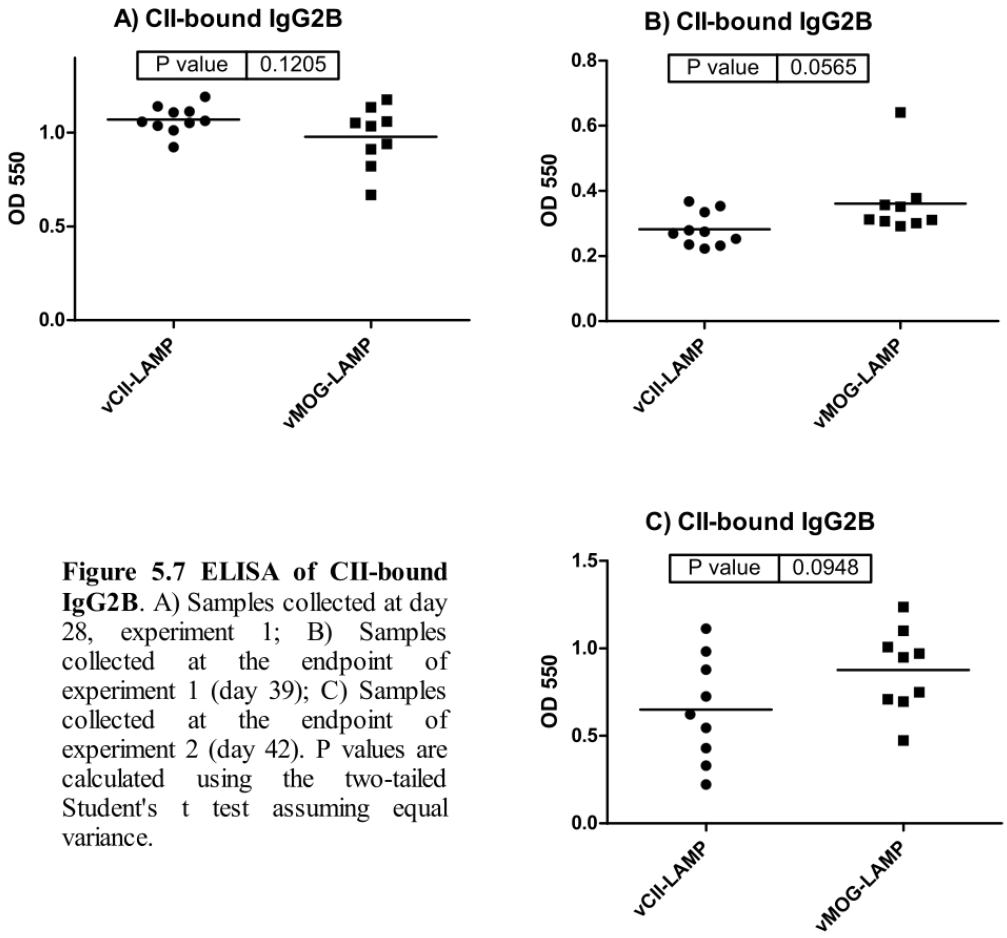


Figure 5.7 ELISA of CII-bound IgG2B. A) Samples collected at day 28, experiment 1; B) Samples collected at the endpoint of experiment 1 (day 39); C) Samples collected at the endpoint of experiment 2 (day 42). P values are calculated using the two-tailed Student's t test assuming equal variance.

Total Ab titres of different serotypes (not CII-specific) were also measured. The availability of mouse Ab standards for these ELISAs meant that absolute values could be extrapolated. No notable difference in total antibody load between the two groups of mice was observed, as illustrated by the total IgG2A and IgG2B counts presented in figure 5.8.

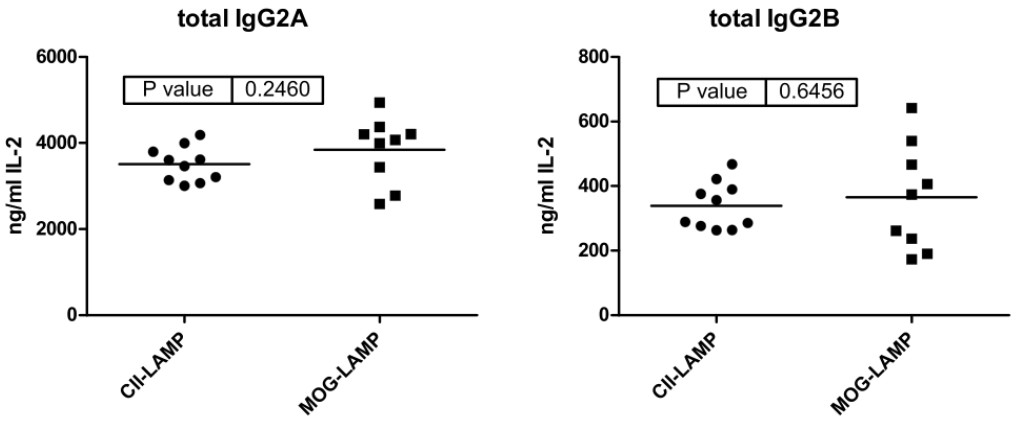


Figure 5.8 ELISA of total IgG2A and IgG2B. Samples collected at the endpoint of experiment 1, diluted and plate-captured using IgG2A-specific antibody or IgG2B-specific antibody respectively. Following incubation with HRP-conjugated detection antibodies, plates were washed and incubated with the BD Biosciences colour-reagent mix. Optic density was measured at 450 nm. P values are calculated using the two-tailed Student's t test assuming equal variance.

5.5.1 *Outliers*

Some of the plots above have points that could be considered outliers. Removing such points can cause the student's t test-calculated significance of the remaining data to increase or reduce depending on the spread of each data-set compared to the difference between the means.

For example, by removing the upper point from the IgM data from day 28, experiment one, the p value increases, because the reduction in spread is outweighed by the reduction in difference-of-means between the groups (fig 5.9). CII-bound IgG datasets provide an example of how removal of a data point can result in an increase in the significance of difference between the groups (figure 5.10)

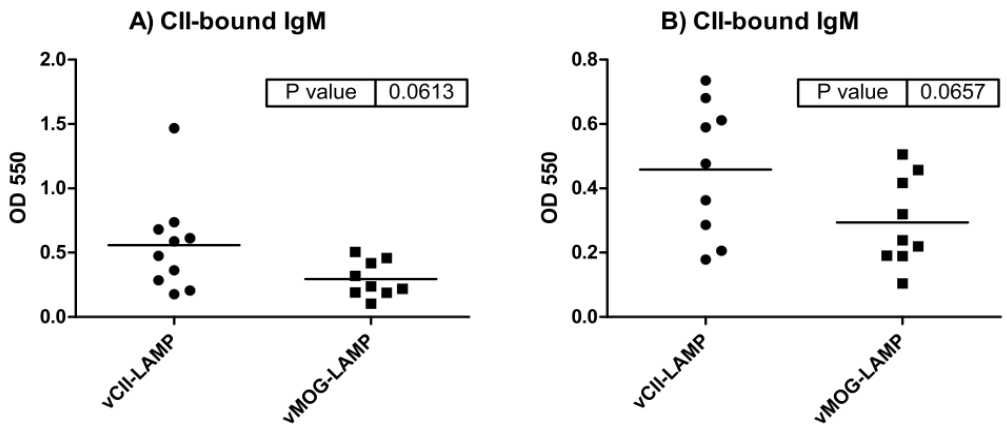


Figure 5.9 Removal of outlier from CII-bound IgM titre, experiment 1, day 28. Before removal of outlying point (A) the student's t test gives a p value of 0.0613 whilst after removal of the outlying point (B) the p value increases slightly to 0.0657.

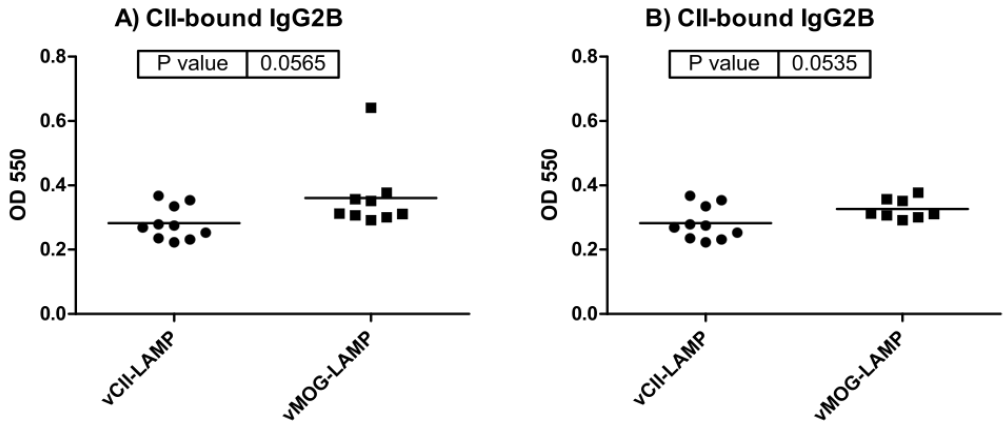


Figure 5.10 Removal of outlier from CII-bound IgG2B titre, experiment 1, day 39. (A) Before removal of the highest value in the vCII-MOG-treated group, the p value is 0.0565. (B) After removal the p value is reduced to 0.0535

Whilst it is sometimes tempting to remove data points that fall at the extremities of a dataset, this should only be done if it is believed that the outlying value has been caused either by a measurement error or by chance that one data point that lies two or more standard deviations from the mean of a normally distributed population has been included in the measured sample. In the cases examined above, neither of these reasons can be assumed. A third reason for the appearance of outliers in a dataset is that the sample is taken from a heavy-tailed population, ie one that is not normally distributed.

Student's t tests are used to evaluate the significance of differences between two sets of continuously variable data each with normal distribution. Whilst a normal distribution is assumed, it is possible that the spread of antibody titres across individual mice is not normally distributed resulting in the outlying points occasionally observed above. Although this argument could hold, the data does appear to broadly fit a normal distribution and with the exception of CII-bound IgG presented in figure 5.5A, a case where the lowest-value data point from the vCII-LAMP-treated group did not even lie very far from the remaining data, removal of outliers does not greatly affect the interpretation of these data.

5.6 *Vector integration number*

Vector integration number was determined by performing qPCR on DNA extracted from the liver, spleen and bone marrow from three mice from each group (experiment 1). Mouse β-actin-complementary primers and WPRE-complementary primers were used to determine cell number and transgene copy number respectively and the data obtained was analysed by an Abi Prism 7000 machine using Sequence Detection software. Transgene copies per cell are calculated by dividing the WPRE-primer-derived value by the β-actin-derived value and multiplying by 2, the number of copies of β-actin per cell. These results are presented in table 5.1.

	LI1	LI2	LI3	LII1	LII2	LII3
β-actin	17848	11151	16911	10403	20415	16090
WPRE	0.55	0.62	0.60	2.20	14.4	5.06
c/c	-	-	-	$4.2x10^{-4}$	$1.4x10^{-3}$	$6.3x10^{-4}$

	BMI1	BMI2	BM3	BMII1	BMII2	BMII3
β-actin	9807	10914	9119	5403	2767	4373
WPRE	2.69	20.11	1.94	1.60	1.02	0.91
c/c	$5.5x10^{-4}$	$3.7x10^{-3}$	$4.3x10^{-4}$	$5.9x10^{-4}$	$7.4x10^{-4}$	-

	SI1	SI2	SI3	SII1	SII2	SII3
β-actin	9659	6543	12854	9862	13366	7623
WPRE	3.67	7.81	131	41.3	7.59	201
c/c	$7.6x10^{-4}$	$2.4x10^{-3}$	0.020	$8.4x10^{-3}$	$1.1x10^{-3}$	0.053

Table 5.1. Quantitive PCR data on vector integration from mouse tissues. DNA was extracted from livers (L), bone marrow (BM) and spleens (S) of three mice from the vCII-LAMP group (group I) or the vMOG-LAMP group (group II). Values for the H_2O controls ranged from 0.5 to 1.1 WPRE. Hence, values below 1 are considered background and not used to calculate a value for transgene copies per cell. Analysis of differences between the groups using the two-tailed Student's t test shows no significant difference between the groups.

Only very low vector copy numbers were detected by qPCR and this did not show a high integration-rate in the liver as expected. These data do, however, appear to confirm that there was no statistical difference in vector integration rates between the two groups of mice.

5.7.1 *Summary*

The data presented in this chapter show the immunological effects of vaccination of male DBA/1 mice with vCII-LAMP as compared to the vMOG-LAMP control *lvv*. The most important result was part-tolerisation to rat CII; the reason that these vectors were designed and made. Mice that were administered with a single *iv* dose of vCII-LAMP 28 days prior to arthritis induction suffered clinical arthritis at an average severity of approximately half that suffered by mice that had been administered the vMOG-LAMP control virus – an effect that was statistically significant. Attempts to elucidate the immunological mechanisms that underlie this effect were also partially successful.

Efforts to measure cytokine production by lymphocytes *ex vivo* were limited by the detection threshold of the ELISA assay and proliferation assays performed on these lymphocytes did not reveal any significant differences between the groups of mice. Measurement of antibody titre, however, did show up differences that were most pronounced in the CII-specific IgG isotypes. IgG, and in particular, the IgG2A and IgG2B are known to correlate with clinical signs of arthritis in mice (Wooley et al. 1985;Banda et al. 2007).

There was an unexpected trend towards higher α-CII titres of all isotypes in the vCII-LAMP-treated mice in samples taken at day 28. This could be because mice treated with vCII-LAMP, having had exposure to the immunodominant $CII_{259-273}$ epitope derived from the transgene, might have been partially primed to respond to immunogens that contain the $CII_{259-273}$ epitope, thus producing an antibody response more rapidly. By the endpoint of the experiments, however, this trend was reversed and α-CII titres in samples taken from vMOG-LAMP-treated animals were significantly higher mirroring their higher arthritic scores. These complementary

data illustrate the partial but significant antigen-specific tolerisation to rat CII that vaccination with the novel *lvv* vCII-LAMP induces.

5.7.2 *Discussion*

The *in vivo* experiments performed in this chapter closely mirrored those from a previous study presented by (Gjertsson et al. 2009). As in that study, mouse numbers were kept to a minimum in the present study. In the previous study two control groups were used, one receiving an *iv* dose of eGFP-expressing *lvv* (LNT-GFP), the other receiving a *lvv* that expressed an unmodified Ii protein including the CLIP domain (LNT-Ii-CLIP). No 'untreated' group of DBA/1 mice was injected with 'mock vector' in either study.

Splenocytes from the LNT-Ii-CLIP-treated group were found to show decreased proliferation compared to splenocytes from LNT-GFP-treated mice both when unstimulated and in response to CII-stimulation, however mice treated with LNT-Ii-CLIP were found to suffer from more severe arthritis than those from the LNT-GFP-treated group. These data suggest that the LNT-Ii-CLIP vector has an immunological effect of its own. This is consistent with other prior studies that found overexpression of the CLIP to have non-specific immunological effects (Rohn et al. 2004). Clearly, this limited the usefulness of LNT-Ii-CLIP as a control. The LNT-GFP control, whilst representing a valid control for administration of functional *lvv* in general, expresses the eGFP protein that is not related to LNT-Ii-CII. Additionally, eGFP itself is known to be immunogenic in some circumstances. For these reasons, a more suitable control was designed for the present study, namely vMOG-LAMP.

Each component of the control *lvv* vMOG-LAMP is identical to that of the vCII-LAMP vector with the exception of the 15-aa $CII_{259-273}$ peptide domain that is replaced by the 12-aa sequence that corresponds to the MOG_{79-90} peptide. Therefore this *lvv* is a control for the $CII_{259-273}$ peptide-loading effect of vCII-LAMP only and does not in any way address the immune effects of *lvv* transduction in general.

Unfortunately, given the limited number of mice available, the effects of *lvv* transduction *per se* on CIA could not be determined.

Comparison of disease score data between the studies actually shows the present study to be less effective at ameliorating arthritis in the 'treatment' group as compared to the control *lvv*-treated mice, although this could be due to bystander suppression effects of vaccination with the vMOG-LAMP control vector. As discussed in section **4.2.4**, the vMOG-LAMP control was designed to eliminate the possibility of non-antigen-specific bystander effects being the mediator of amelioration of CIA. This is because vMOG-LAMP would deliver and load the MOG_{79-90}-peptide on MHCII-I-A^q molecules in the same way that vCII-LAMP loads the $CII_{259-273}$ peptide for MHCII-I-A^q-presentation. Subsequent recognition, by MOG_{79-90}-responsive T cells in the absence of costimulatory signals, could result in tolerogenic immune effects that could non-specifically reduce inflammation as outlined in section **1.1.3**. Unfortunately, as MOG_{79-90}/I-A^q-responsive hybridomas are not available, as in other studies (Batsalova et al. 2010), the presence of these complexes had to be assumed and could not be shown. Nevertheless, expression of the eGFP-tagged fusion proteins was inferred by the presence of fluorescence in transduced cells.

Measurement of TGF-β production by splenocytes from culled mice in the previous study, either without stimulation or in response to restimulation with CII, revealed a significantly higher production in cells taken from LNT-Ii-CII-treated mice compared with cells from LNT-Ii-CLIP-treated mice (Gjertsson *et al*. 2009). The elevated levels of TGF-β production was taken to indicate a skewing to a regulatory phenotype of those splenocytes. Equivalent experiments were performed during the present study, both on splenocytes and draining lymph node cells. The stimulations themselves were kindly performed by Inger Gjertsson in Gothenburg with the supernatants being sent to UCL, UK for cytokine measurement ELISAs. Unfortunately TGF-β levels were not detectable above background in these samples. IL-10 and IL-17 were detected above background only in those samples stimulated

with Con A (the positive control) and levels detected in samples obtained from splenocytes were substantially lower than those measured in draining lymph node samples. Although the samples were shipped 'on dry ice' it is possible that there was a significant degradation of the cytokines within the samples between the time when they were harvested in Gothenburg and the time when the cytokine concentration was measured in London.

Another consideration for similar studies in future might be to assay levels of cytokines from blood samples during the study to try to detect changing levels during disease progression. This could give an indication as to which cytokines are mediating disease or therapeutic effects.

Many antibody subtypes were measured in the present study. The difference in antibody titre between the two groups was significant only at the end-point (day 42) of the second experiment in the cases of CII-specific IgG2A and total CII-specific IgG. These data resemble those presented by Gjertsson *et al*. that show CII-specific IgG2A levels to be significantly lower in LNT-Ii-CII mice compared to LNT-Ii-CLIP-treated mice.

Together, the data on the biological effects of *iv* vaccination of DBA/1 mice with vCII-LAMP presented in this chapter can be interpreted as showing the vCII-LAMP vector as having a similar efficacy to LNT-Ii-CII in reducing anti-CII IgG2A titre and ameliorating CIA.

Chapter 6. Results IV
Further applications

6 Further applications

6.1.1 *Introduction to the need for an LH3-expressing vector*

The reasons behind re-designing the $CII_{259-273}$-delivering fusion proteins, using LAMP- or DM-based chimeras rather than Ii-based chimeras used previously were twofold (Gjertsson et al. 2009).

Ii comprises one part of the trimer that forms the MHCII complex, the other polypeptide chains being I-Aα and I-Aβ (see figure 1.1). Firstly, replacing Ii as the delivery molecule eliminates the risk of problems that might be caused by non-physiological over-expression of a constituent part of the MHCII complex.

Secondly, endogenous CII is heavily post-transcriptionally modified through hydroxylation and glycosylation by enzymes such as LH3 (Wang et al. 2009;Schegg et al. 2009). These modifications are known to occur at the lysine-264 residue and this is important for the immunogenicity of the $CII_{259-273}$ peptide (Corthay et al. 1998;Corthay, Backlund, & Holmdahl 2001). The degree of glycosylation of the $CII_{259-273}$ peptide that was presented by APCs transduced with the Ii-chimera was low as measured by the strong response of those CD4+ hybridoma lines responsive to the naked peptide (eg HCQ4) and the poor response of glycosylated-peptide-responsive lines (Gjertsson et al. 2009). It was thought that this could be because access of the glycosylation machinery to the nascent peptide might be limited by the fact that, as the $CII_{259-273}$ peptide directly replaced the Ii CLIP domain, the Ii-chimera would fold into the MHCII complex, embedding the $CII_{259-273}$ peptide in the peptide-binding groove before significant post-transcriptional modifications could occur. One solution, developed by James Devitt, was instead to fuse the $CII_{259-273}$ peptide to the carboxyl terminus of Ii, forming a *lvv*-expressed construct, known as SICW (*sffv*-Ii-$CII_{259-273}$-WPRE). This approach did result in greater glycosylation of the presented $CII_{259-273}$ peptide but brought with it the drawback of concomitant overexpression of the CLIP domain that is known to have non-specific, undesirable immunological effects (Rohn et al. 2004). It was hoped that by using the construct

design in the present study, namely a short TM domain from LAMP-1 or HLA-DM fused to the $CII_{259-273}$ peptide, greater access might be afforded to the glycosylation machinery resulting in a greater overall degree of glycosylation of the construct-derived peptides. This, however, was not borne out by the antigen presentation data presented in chapter 4.

LH3 is expressed globally during mouse embryo development and in most tissues either intra- or extra-cellularly (Salo et al. 2006). Procollagens are modified by LH3 within the lumen of the ER. It has been shown that eGFP-tagged LH3 (that was functional by exhibiting glucosyltransferase activity) was found predominantly in the ER, and also in the Golgi of transfected COS-7 cells (Heikkinen et al. 2000).

It was hypothesised that co-expressing LH3 with the fusion constructs may enhance glycosylation. The cDNA of LH3 was cloned into the pLNT backbone and the *lvv* denoted vLH3cor63 was prepared.

6.1.2 *Expression of LH3 – titration of vLH3cor63 by qPCR*

Titres of *lvv* that express eGFP-tagged fusion proteins were determined by flow cytometry but this was not possible for vLH3cor63. Infectious titre of vLH3cor63 was determined by treating wells containing 100,000 293T cells with serial dilutions of vLH3cor63 before harvesting cell DNA on day 3 and measuring the number of integrated vector by qPCR using WPRE-specific probe and primers (figure 6.1).

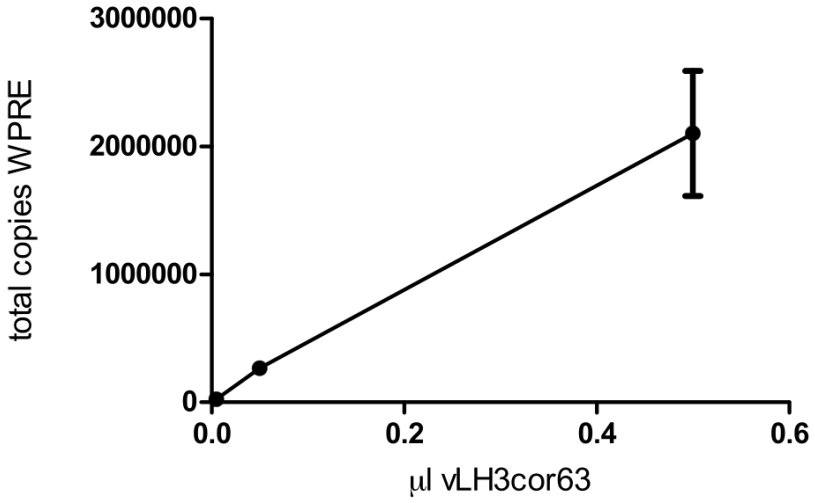


Figure 6.1 Titre of infectious units of vLH3cor63 by qPCR . Values obtained using dilutions corresponding to 5 x 10^{-3} µL, 5 x 10^{-2} µL and 0.5 µL are included; those values corresponding to 5 µL and 50 µL are excluded because they fall outside the linear range. A titre of 4 x 10^{9} infectious units per mL was calculated.

To control for differences in the sensitivity between flow cytometry and qPCR in detecting transgenes in a target cell, qPCR of vSEW of known titre of 10^{9} was performed alongside the titre of vLH3cor63 shown in figure 6.1. Analysis of infectious titre by qPCR of the vSEW preparation generated a calculated value of 5 x 10^{9} infectious units per mL – five-fold greater than the value calculated by flow cytometry. This was repeated also using vCII-DM and a five-fold discrepancy was consistently found. Hence for calculating experimental volumes and MOI's, where the value for infectious titre was only available from qPCR, this value was reduced by 5-fold to achieve equivalent MOI's.

Although the sequence of LH3 cDNA in the pLNT backbone was confirmed by sequencing and infectivity of the vector was confirmed by qPCR, protein expression could not be verified by fluorescence so western blot analysis was used to detect LH3 expression by transduced cells.

6.1.3 *Expression of LH3 – detection of LH3-expression by western blot*

Plates were seeded with 100,000 293T cells that were then transduced with vLH3cor63 at an MOI of 25 and cultured for a further 4 days, passaging when necessary. Cells were then harvested, lysed in reducing sample buffer and run on a polyacrylamide gel in reducing conditions. After transfer onto a polyvinylidine membrane, samples were probed with antibodies reactive to human β-actin or diluted anti-serum from rabbits immunised with LH3 (Ruotsalainen et al. 2006b) (figure 6.2). It was necessary to expose the developing polyvinylidine difluoride membrane that had been probed with LH3-reactive sera for 30 minutes due to the weakness of the signal.

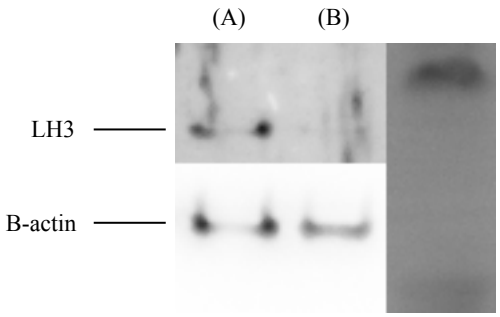


Figure 6.2 Western blot of transduced 293T cells with vLH3cor63.
(A) Transduced with vLH3cor63;
(B) Untransduced. *Top panel*: membrane probed with LH3-reactive sera; *bottom panel*: membrane probed with β-actin Ab; *right-hand panel*: pre-stained phosphorylase (above) and bovine serum albumin (below).

The calculated molecular mass of LH3 is approximately 85 kDa. A band of expected size is visible in the lane containing lysate from 293T cells transduced with vLH3cor63 (figure 6.2 lane A) but not in the sample lane from untransduced cells (figure 6.2 lane B).

6.1.4 *Co-expression of LH3 in 3T3-I-A^q cells transduced with vCII-LAMP results in glycosylation of the CII-$_{259-273}$ peptide*

The functional assay performed with the vLH3cor63 vector was to co-transduce the cell line with vLH3cor63 together with vCII-LAMP, vSICW (the vector expressing the $CII_{259-273}$ peptide fused to the C-terminus of Ii) or vCII-MOG

and compare the antigen presentation data with that from 3T3-I-A^q cells transduced with FP-*lvv* alone. These data are presented in figure 6.3.

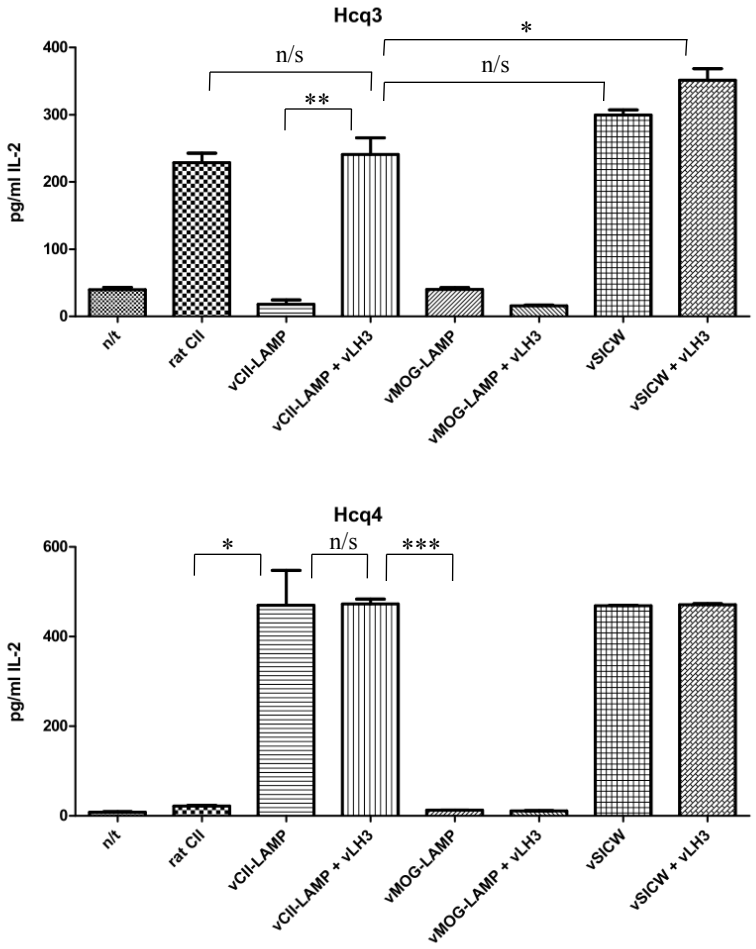


Figure 6.3 Antigen presentation of CII$_{259-273}$ by 3T3-I-A^q cells transduced with vLH3cor63, vCII-LAMP and vMOG-LAMP. HCQ3 responds to rat CII (heavily glycosylated) and to 3T3-I-A^q cells co-transduced with vCII-LAMP and vLH3cor63 but not to cells transduced with vCII-LAMP alone. HCQ4 responds to unmodified CII$_{259-273}$ peptide and in this case is stimulated by both vCII-LAMP- and vSICW-treated cells, singly transduced or cotransduced with vLH3cor63.

P values calculated by 2-tailed Student's t test: *** denotes $p < 0.001$; ** denotes $p < 0.01$; * denotes $p < 0.5$; not significant (n/s) denotes $p > 0.05$

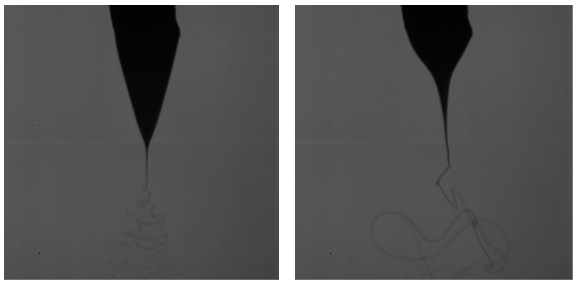
The data presented in figure 6.3 shows that by using *lvv* to express LH3 at the same time as the $CII_{259-273}$-containing CII-LAMP, the $CII_{259-273}$ peptide is glycosylated to a significant degree before being presented.

6.1.5 *Summary of vLH3cor63*

In this chapter, generation of infectious LH3-expressing *lvv* has been demonstrated. It was also shown that such *lvv*-mediated expression of LH3 could be used to enhance O-glycosylation of lysine residues, in particular the lysine-264 of the $CII_{259-273}$ peptide, present in both the CII-LAMP construct and the Ii-fusion SICW.

6.2.1 *Introduction to Bio-electrospray*

Bio-electrospray (BES) is a jetting technique for the direct physical manipulation of living cells (Jayasinghe 2007). Developed by Dr Jayasinghe at the department of engineering, UCL, BES and the closely related technique of Cell Electrospinning (CE) pass cell suspensions through an electrically charged large-bore needle such that the solution is attracted to a target at the ground electrode (figure 6.4). From the droplet at the tip of the needle, a jet evolves that subsequently fragments into droplets the size and distribution of which can be controlled by varying the applied potential difference, the flow rate and liquid properties (Ganan-Calvo & Gordillo 2001). If the cell suspension that passes out from the needle is an alginate solution of sufficient concentration, the cells are unable to egress from the resulting cell droplets (or other structures). When this technique was first applied to living cells, the viability of those cells were found to be unaffected, and it has since been applied to different cell types including neurons (Eagles, Qureshi, & Jayasinghe 2006), stem cells (Mongkoldhumrongkul, Flanagan, & Jayasinghe 2009) and even whole organisms (Clarke & Jayasinghe 2008;Geach et al. 2009).



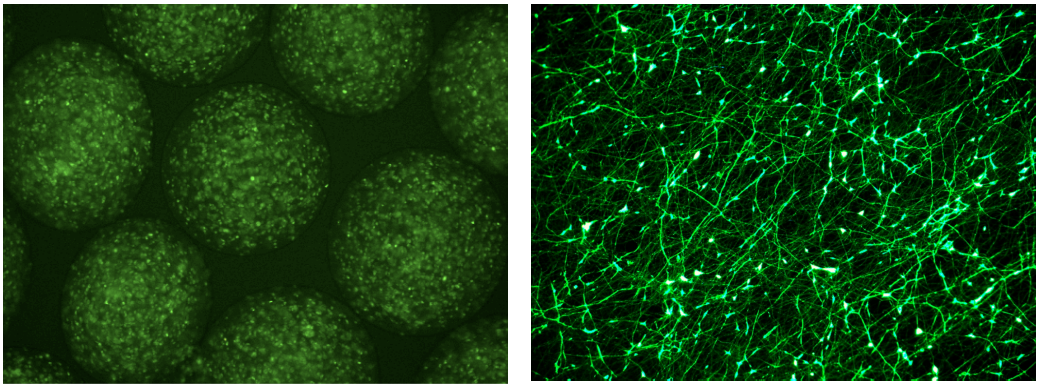
A) B)

Figure 6.4 Electrospray of cellular suspensions in stable conditions in A) BES; at higher voltages, a narrower band of medium is ejected from the needle, and B) CE; at lower voltages the jet is spead more widely.

6.2.2 Combining Bio-electro spray with gene therapy

In collaboration with Dr Jayasinghe we have for the first time shown that cells can be genetically manipulated using *lvv* before being physically manipulated through BES or CE to form multicellular arrangements of gene-modified cells

(figure 6.5). More specifically, 3T3-I-Aq cells were transduced with vCII-LAMP and processed using BES or CE to form multicellular structures of cells that express the eGFP-containing fluorescent fusion protein CII-LAMP. The result of changing the flow parameters to switch from BES to CE is that instead of forming cell-bearing droplets of near-uniform size, cell-bearing threads of different thicknesses can be generated. These techniques were each applied to 3T3-I-A^q cells that had been transduced with vCII-LAMP and suspended in alginate (figure 6.5).



A) B)

Figure 6.5 Flourescent micrographs depicting structures formed from 3T3-I-A^q cells treated with vCII-LAMP generated by way of A) BES and B) CE.

The aim of this work was to show that cells were not only viable after BES but were also able to retain the gene therapy-derived function, in this case antigen-presentation of the $CII_{259-273}$ peptide. This was assessed through co-culture of bio-electrosprayed cells with HCQ4 T cell hybridomas followed by measurement of the concentration of secreted IL-2 by sandwich ELISA. Processing the cells using BES resulted in only a small reduction in antigen-presentation potency (figure 6.6) although this reduction was significant when compared to the culture control ($p = 0.047$).

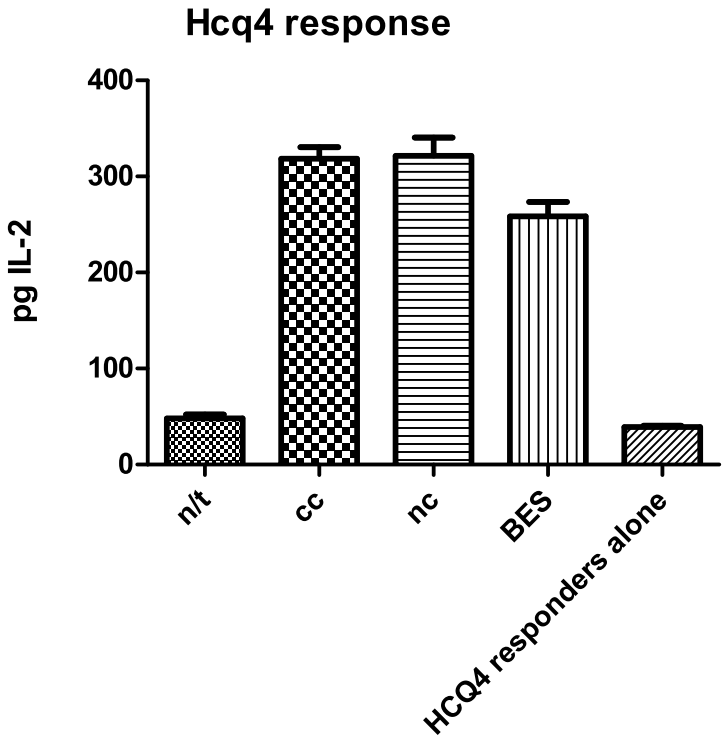


Figure 6.6 HCQ4 response to vCII-LAMP-treated 3T3-I-A^q cells after bio-electrospray (BES), passage through an un-charged needle (needle control, nc), culture control (cc), or in response to untransduced 3T3-I-A^q cells (n/t). 2-tailed Student's t test, bes vs cc, p = 0.047.

6.2.3 *Summary of bio-electrospray*

In this part of the project BES was shown to physically distribute genetically-modified cells and it was evident that these cells retain their gene-therapy-acquired characteristics following BES. This means that they retained the ability to express fluorescent fusion protein and present the immunodominant $CII_{259-273}$ epitope on MHCII molecules. This work could enable studies of the best location within an animal for tolerogenic antigen expression. Multicellular bodies of APCs could be implanted into experimental animals at locations such as beside the spleen, lymph node or other organs. There may be more tolerogenic or more immunogenic locations.

6.3 *Discussion*

This chapter has investigated the possibility of combining the *lvv* developed and tested in the previous chapters with other gene therapy *lvv* (the LH3-expressing vLH3cor63) or with biotechnology elements in development for tissue engineering (BES). It was found that the *lvv* that induce expression of the novel fusion proteins described in this thesis, when used in concert with these other vectors or technologies, were able to induce responses from CD4+ T cell hybridomas that were predicted and hoped for.

When used with BES to produce multicellular bodies of antigen-presenting 3T3-I-A^q cells, the response from cocultured responder hybridomas was only slightly lower than the response to non-BES-processed controls. This opens avenues for future work including the generation of layered multicellular structures of different cell types to investigate or mimic the types of immunological cell-cell interactions that occur in lymph nodes and other immune tissues and organs.

When used with the LH3-expressing *lvv* vLH3cor63 the hybridoma response changed in precisely the way that was hoped. By exogenously co-expressing the key enzyme in the glycosylation process, LH3, alongside the novel fusion protein CII-LAMP, the hybridoma line HCQ3 response was raised to a level not significantly different to that in response to 3T3-I-A^q cells presenting rat CII. This represents the achievement of one of the main goals of the design of this study, namely to generate a *lvv*-mediated system for driving presentation of $CII_{259\text{-}273}$ peptides, a significant proportion of which appeared to be glycosylated. Whilst the original project design aimed to achieve this through simple expression of the fusion proteins, it was eventually found to be necessary to co-express LH3 with the fusion proteins in this study to attain levels of $CII_{259\text{-}273}$ peptide glycosylation detectable with CD4+ hybridomas.

This result is, to the best of our knowledge, the first time *lvv* co-transduction with a glycosylating enzyme has induced detectable substrate glycosylation. However, this data was obtained in a 3T3-derived cell line only and was not

confirmed in mBM-DCs. Generation of a bicistronic *lvv* that expresses both the fusion protein CII-LAMP and LH3 could form the next stage in further investigation of the use of *lvv*-driven presentation of glycosylated $CII_{259-273}$ peptides.

Chapter 7. General discussion

7 General discussion

7.1 *Achievements*

The current project has demonstrated several novel applications of existing principles. Transduction of APCs with FP-*lvv* was shown to result in fusion protein-derived peptide presentation on MHCII molecules confirming *lvv* as a means to achieving peptide-antigen presentation. Furthermore, it was shown that direct injection of such FP-*lvv* into the tail veins of DBA-1 mice results in protection from arthritis that manifests as significant amelioration of disease at time-points of over nine weeks after *lvv* injection. This represents an improvement over previous studies and also a success for rational design in novel therapeutics.

The previous studies that this work has improved upon used rational designs to induce MHCII-presentation of the $CII_{259-273}$ peptide, immunodomminant in CIA, either by replacing the CLIP domain of Ii with the $CII_{259-273}$ peptide (Gjertsson et al. 2009) whilst a second, similar study fused the $CII_{259-273}$ peptide to the luminal, C-terminus of Ii, in the fusion construct denoted SICW. Other groups have used other retroviral vectors to express Ii-based chimeras that induce tolerance to other peptide antigens, namely the MOG_{40-55} peptide (Eixarch et al. 2009). The current project has rationally designed fusion proteins that deliver a peptide epitope for MHCII presentation not through conjugation to Ii, a constituent part of the MHCII trimer that folds within the ER, but simply through conjugation with short TM/cytosolic tail motifs from either of two other intracellular proteins, LAMP-1 and H2-DM that are known to traffic down the endosomal pathway. Hence, instead of inducing $CII_{259-273}$ presentation through direct targeting to the MHCII complex this project has achieved it through simple intracellular trafficking.

Expression of the fusion proteins CII-LAMP or CII-DM in primary mBM-DCs after transduction with equal infectious titres of *lvv* vCII-LAMP or vCII-DM allowed comparison of the two trafficking domains. Most importantly, these fusion proteins were compared for their capacity to induce surface presentation of the

$CII_{259\text{-}273}$ peptide to MHCII-restricted T cell hybridomas. CII-LAMP-expression by the mBM-DCs resulted in stronger stimulation of the responding hybridomas leading to the conclusion that conjugation to the LAMP-1 TM/cytosolic domain is more efficient in inducing MHCII-loading of the $CII_{259\text{-}273}$ peptide. When vCII-LAMP was administered to DBA/1 mice *iv*, 28 days prior to CIA induction, it caused significant amelioration of disease and a significant reduction in CII-specific IgG at the time when the experiment was terminated. This is the first time *lvv* have been used to induce tolerance as a result of expressing such LAMP-l/peptide antigen chimeras.

The fusion proteins CII-LAMP and CII-DM are not expected to form complexes with MHCIl molecules in the way that Ii-based fusions might and so it was hoped that this might afford the glycosylation machinery greater access to the $CII_{259\text{-}273}$ peptide domain of CII-LAMP and CII-DM giving rise to greater glycosylation than the modest levels presented by Gjertsson *et al* in their supplementary table. The prospect of enhanced glycosylation of the lysine-264 residue was one of the key motives for designing novel $CII_{259\text{-}273}$ peptide-delivering FP-*lvv*. However, when antigen-presentation data from T cell hybridomas reactive to $CII_{259\text{-}273}$ peptide in the naked, unmodified form (HCQ4) or glycosylated forms (HCQ3) were compared with data obtained from APCs expressing vCII-LAMP, vCII-DM or the SICW construct (that fuses the $CII_{259\text{-}273}$ peptide onto the C-terminus of Ii) presented $CIl_{259\text{-}273}$ peptide on cells transduced with the two novel FP-*lvv* were shown to be glycosylated to a much lesser extent (an example of this is shown by the data obtained from HCQ3 response to 3T3-I-A^q cells transduced with vCII-LAMP or vSICW in figure 6.3). To enhance the glycosylation of the $CII_{259\text{-}273}$ peptide domain of CII-LAMP, LH3 was cloned into the *lvv* backbone plasmid and the vLH3cor63 vector was prepared.

Capable of hydroxylation, galactoslytransferase and glucosyltransferase activity (Heikkinen et al. 2000;Wang et al. 2002) LH3 is able to modify collagen of different types (Wang, Valtavaara, & Myllyla 2000). LH3 knock-out in mice is lethal due to abnormal collagen type IV distribution preventing basement membrane

formation (Ruotsalainen et al. 2006c). Expressed globally in embryonic mouse tissues, LH3 is also detected at low levels in a broad range of adult mouse organs both intracellularly and extracellularly although which cell types express LH3 is not known (Salo et al. 2006). Presumably, given the very low levels of glycosylated $CII_{259-273}$ presented by DCs transduced with both the *lvv* described in this study and those by Gjertsson *et al*, LH3 expression in DCs at levels too low to efficiently glycosylate the nascent fusion proteins. The present study found that by using the *lvv* vLH3cor63 to co-express LH3, the glycosylation state of the $CII_{259-273}$ peptide presented by 3T3-I-A^q cells co-transduced with vCII-LAMP is greatly enhanced (figure 6.3). To the best of our knowledge this is the first time that gene-delivery of a glycosylating enzyme has been shown to effect a detectable increase in the glycosylation state of specific amino acid residues.

Another notable finding was the difference in antibody titres between sera from mice vaccinated with vCII-LAMP and vMOG-LAMP. At the earliest time-point at which samples were collected, day 28 of experiment 1, the trend of higher Ab titres in the vCII-LAMP group compared to the vMOG-LAMP group was unexpected, but observed in every measurement of CII-specific antibody titre. As described in section **1.2.2** *Antibody response*, antibody titre does not always correlate with disease - indeed, mouse strains refractory to CIA secrete high titres of anti-CII antibodies in response to rat CII emulsified in CIA. The trend towards higher concentration of anti-CII Ab isotypes in mice vaccinated with vCII-LAMP at day 28 could be attributed to the earlier sub-immunising exposure to the immunodominant $CII_{259-273}$ peptide from the vCII-LAMP vector. Although ultimately having the effect of reducing the immune response to the immunising rat CII, as this effect is presumed to be due to a sub-immunogenic priming to the $CII_{259-273}$ epitope, it is conceivable that this also has the effect of reducing the time between rat CII-exposure and production of measurable titres of CII-specific Ab.

Important in the pathogenesis of CIA are high titres of IgG, in particular the complement-fixing isotype IgG2A. Hence the titres of these isotypes were separately

measured. However, it was found that only the total CII-specific IgG was significantly lower in vCII-LAMP mice when compared to mice vaccinated with the vMOG-LAMP construct. Whilst the lack of significant differences in IgG2A precludes a statement on the mechanism by which vCII-LAMP exerts its protective action, the significant reduction of anti-CII IgG is a piece of key biological data that correlates with the reduced clinical score. Although the three time-points observed by this study - immediately at disease onset, and at the point of termination of each experiment 1 and 2 - are important, perhaps the best way to examine the relationship between tolerance induction, Ab production and disease progression would be to perform measurements upon the Ab concentration of sera collected at time-points of every two or three days throughout the experiment. Such an undertaking was not considered possible within the context of this project.

7.2 *Limitations*

Whilst the discovery that the novel FP-*lvv* described in this thesis do significantly ameliorate clinical scores in CIA constitutes the accomplishment of primary goal of this project, cellular interactions that must underlie the significant shift in the immunological behaviour of the group of mice vaccinated with vCII-LAMP were not within the scope of the project and thus not fully elucidated. Interactions between the MHCII-$CII_{259-273}$ complex on transduced APCs with T cell hybridomas were demonstrated in vitro as being dependent upon transduction with $CII_{259-273}$-delivering FP-*lvv* such as vCII-LAMP, vCII-DM or vSICW. Whilst it is assumed that similar interactions between transduced I-A^{q}+ APCs and CD4+ T cells are behind the antigen-specific tolerogenicity of these vectors, this was not demonstrated with cells from vCII-LAMP-treated mice. It was thought that the low numbers of transduced cells in any one tissue following *iv* administration would certainly preclude the induction of any detectable response from coculturing tissue-derived cells with T cell hybridomas *ex vivo*. That mBM-DCs, transduced ex vivo, are able to strongly stimulate CD4+ T cell hybridomas is viewed as a good

indication that transduction of APCs or APC progenitors *in vivo* would result in anergising or otherwise tolerising interactions with $CII_{259-273}$-reactive CD4+ T cells that reduce the animal sensitivity to immunisation with rat CII. It would be extremely difficult to detect such transduced cells from mice on the basis of fluorescence - even qPCR was only able to detect counts of vector-derived WPRE from mouse tissues just above background.

Assessment of the precise reactions within experimental mice was never a stated aim of this study, however cellular responses using spleen or lymph node cultures to assess the differences in proliferative and cytokine-production responses between the groups of mice were sought once it was shown that the vCII-LAMP vector had a significant protective effect. Neither, however, revealed significant differences between the groups of mice, although, in the case of induced production of TGF-beta, IL-10 and IL-17 in response to rat CII, the cytokine levels were broadly below the threshold of detection.

The only significant biological data taken from the mice in this study was the difference in CII-specific IgG. Whether this can be interpreted as indicating that CIA is Ab-mediated, however is unclear. It is known that B cell-deficient mice are unable to develop CIA, so the difference in IgG titre is highly relevant in pathogenesis. The mechanism by which this value is reduced has not been directly elucidated. The importance of CD4+ T cell help in antibody switching and the $CII_{259-273}$-presentation by transduced APCs in vitro strongly suggests that reduced anti-CII IgG is a downstream effect of tolerogenic antigen presentation.

Arguably, a limitation of this study and others like it, inherent in their design, is the limited similarity of an animal model to the human condition that it represents. Of course this is also true of CIA in DBA/1 mice. Other mouse models include AIA, CAIA and autologous CII-induced arthritis, discussed in section **1.2** and reviewed in (Williams 1998). Each also has disadvantages. Susceptibility of mouse strains to CIA is dependent on MHCII type, a similarity to the correlation of susceptibility to

RA with HLA-DR1 and HLA-DR4. Indeed, the crystal structure of HLA-DR4 in complex with the human $CII_{257-273}$ has been solved (Hurlbert & Izard, 2002).

Whilst the MHCII-linked susceptibility is an advantage CIA has over models such as CAIA, the lack of MHC diversity in inbred mice is an obvious limitation of using such animals as models for human conditions. Greater diversity, however, would limit the repeatability of experimental data and in turn limit the usefulness of a model that may be of a closer likeness to human RA. Such reasons limit the usefulness of autologous CII-induced arthritis - disease progression varies greatly and many mice do not show signs of clinical arthritis at all. Hence, although heterologous CII-induced CIA as used in this study is the most widely used model, as with all animal models of human disease, it is not a perfect model. This, along with the safety issues associated with *lvv* do limit the transferability of this type of therapeautic strategy into human patients. Nonetheless, this study does present significant additional insights as to the use of exogenously expressed, endosomally targeted fusion proteins in the induction of antigen specific tolerance.

7.3 *Future work*

One notable achievement of this study was the successful use of gene therapy-type methods to deliver the post-translationally modifying LH3 enzyme to enhance the glycosylation of the lysine-264 residue of the $CII_{259-273}$ peptide presented by transduced 3T3-I-Aq cells. This represents a novel finding in itself but, furthermore, could be important in developing gene therapy protocols to induce tolerance to CII. When presented within the context of soluble MHCII molecules the $CII_{259-273}$ peptide was only able to protect mice from CIA in its galactosylated form (Dzhambazov et al. 2006).

The data showing enhanced glycosylation following cotransduction with vLH3cor63 along with vCII-LAMP represents preliminary data on the use of LH3-encoding vectors. In the context of the present study, the next question to be asked was whether similar results would be obtained in co-transduced mBM-DCs (or other

primary cells). Secondly, before translating this approach into the mouse model, a strategy for ensuring APCs *in vivo* would be transduced in such a way that both transgenes would be expressed in the same cell must be addressed. Both of these problems were approached although neither was completed within the available time. Initial attempts to cotransduce mBM-DCs proved technically challenging and erratic probably owing to the relatively low efficiency of the mBM-DC co-transductions per individual cell.

Cotransduction of mBM-DCs or other primary APC *ex vivo* is one way to ensure a high proportion of APCs are co-transduced and will express both LH3 and CII-LAMP as long as transductions are performed at relatively high MOIs. Such cells could then be infused into syngenic mice and assessed for their tolerogenic effects. Administration of each vector *iv* would be insufficient to be assured of a useful rate of co-transduction - only a very small population of cells are likely to be infected by both vectors. The only way in which an *iv* transduction protocol, similar to that used previously in this study, might be used would be if a single bicistronic vector expressing both LH3 and CII-LAMP could be made. (Significant time was spent attempting to clone LH3 into a vector backbone containing the *sffv* promoter and an adjacent minimal CMV promoter that had had the cDNA encoding CII-LAMP inserted beside it. Unfortunately, clones containing LH3 could not be obtained). Together with Inger Gjertsson's collaboration in Gothenberg, these studies will be continued.

Finally, the implications that this study has for future clinical trials in humans will be considered. The safety aspects of current *lvv* clearly preclude the use of vectors such as vCII-LAMP for use in non-lethal human diseases such as RA. The work presented here is, nonetheless, of interest to researchers planning future studies of novel therapeutic protocols for the treatment of autoimmune diseases. Firstly, it has been demonstrated that administrating vectors *iv* that express rationally-designed endosomal-targeted peptide-antigen-containing fusion proteins can have beneficial effects by reducing the severity of subsequent responses to that antigen. There is no

reason to believe that this effect would be limited to either the delivery vectors used in this study (integrating *lvv*) nor to the model peptide antigen tested here. Whilst expression levels obtained from using non-integrating *lvv* are often significantly lower (Karwacz et al. 2009) than from integrating *lvv* and, currently, attaining sustained expression after multiple rounds of cell replication appears distant, these problems are not necessarily insurmountable. Furthermore, it may prove that for effective, antigen specific modulation of immune disorders, continuous antigen presentation may not be required. A pulse of tolerogenic peptide presentation may effectively prime the immune system to reduce the severity of subsequent responses to that antigen. A study of the fusion constructs described here, expressed by non-integrating *lvv* administered *iv* to DBA/1 mice would be an obvious starting-point.

It may be that efficient priming of the immune system of the nature described here does require the high-level and sustained expression profiles currently only achievable with integrating vector technologies. Even if this is the case, the antigen-presenting strategies presented in this thesis might still find application in the treatment of human disease. Life-threatening conditions such as cancer and HIV infection would often be much more amenable to treatment if only the host immune system were able to recognise and effectively respond to tumour- or virus-associated antigens. Whilst MHCII-presentation in this study has acted to increase tolerance to the presented antigen, some groups have already investigated co-expression of DC-activating proteins alongside model antigens to enhance CD8 T cell responses to the antigen (Rowe 2009). A similar strategy, if used alongside MHCII-directing fusions such as CII-LAMP might act to immunise CD4 T cells rather than tolerise them.

Acknowledgements

None of the work presented in this thesis would have been possible without the ceaseless encouragement, kind advice and fatherly guidance of Kenth Gustafsson, my supervisor. Not only did his interest in my development stretch beyond the laboratory, he also made it possible for me to collaborate with Inger Gjertsson's group at the Department of Rheumatology, University of Gothenburg, without which my in vivo studies would not have been possible. Each member of the Molecular Immunology Unit at the Institute of Child Health has helped me along the way, be it assistance in setting up a Western Blot or a friendly arm around my shoulder to keep my blood from boiling as I stared at yet another agar plate devoid of colonies. To name some but not all would risk devaluing the irreplaceable support and unique atmosphere at the MIU to which everyone has contributed and which I deeply appreciate. I cannot, however write these thanks without specifically mentioning a few names. Albert actually got me to smile after two weeks of work culminated in a load of dead cells (no mean feat) while Shahla, Conrad, Luisa, João and João are irreplaceable and incredibly genuine friends whose daily kindness and football-banter will be greatly missed. Siobhan, Austin, Mike and Gerben advised me on matters of antibodies, marathons and motorbikes. Adrian was a friendly and encouraging second supervisor while Sue ensured the ICH actually ran, but rarely ran out of anything. Natalie is my fellow Ward and Californian travel-buddy while Emma is my fellow Gooner and the closest thing I ever had to a big sister. Jim welcomed me to Team Gustafsson and his smiling streams of expletives will never be forgotten. The time I spent with Roman working on special projects outside the lab unquestionably saved me from complete insanity as I toiled in the thesis-mire. The biggest thankyou goes to Yin for maintaining a calm, pragmatic approach to post-graduate life whenever the fan got hit. He is my brother. My housemates at Hilldrop Road have been a family to me to the extent that they put up with me being a miserable sod at times and never held it against me. But it is the unconditional love of Elaine, Steve and Debbie that has meant that I could get this far in the first place. Because of them, everything is possible.

References

Aaltonen, J., Bjorses, P., Perheentupa, J., Horelli-Kuitunen, N., Palotie, A., Peltonen, L., Lee, Y. S., Francis, F., HenningSteffen, Thiel, C., Leharach, H., & Yaspo, M. L. 1997, "An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains", *Nat Genet*, vol. 17, no. 4, pp. 399-403.

Abdelnour, A., Arvidson, S., Bremell, T., Ryden, C., & Tarkowski, A. 1993, "The accessory gene regulator (agr) controls Staphylococcus aureus virulence in a murine arthritis model", *Infect.Immun.*, vol. 61, no. 9, pp. 3879-3885.

Abdul-Majid, K. B., Jirholt, J., Stadelmann, C., Stefferl, A., Kjellθn, P., Wallstr÷m, E., Holmdahl, R., Lassmann, H., Olsson, T., & Harris, R. A. 2000, "Screening of several H-2 congenic mouse strains identified H-2q mice as highly susceptible to MOG-induced EAE with minimal adjuvant requirement", *Journal of Neuroimmunology*, vol. 111, no. 1-2, pp. 23-33.

Adriaansen, J., Khoury, M., de Cortie, C. J., Fallaux, F. J., Bigey, P., Scherman, D., Gould, D. J., Chernajovsky, Y., Apparailly, F., Jorgensen, C., Vervoordeldonk, M. J. B. M., & Tak, P. P. 2007, "Reduction of arthritis following intra-articular administration of an adeno-associated virus serotype 5 expressing a disease-inducible TNF-blocking agent", *Annals of the Rheumatic Diseases*, vol. 66, no. 9, pp. 1143-1150.

Agarwal, R. K., Kang, Y., Zambidis, E., Scott, D. W., Chan, C. C., & Caspi, R. R. 2000, "Retroviral gene therapy with an immunoglobulin-antigen fusion construct protects from experimental autoimmune uveitis", *J Clin.Invest*, vol. 106, no. 2, pp. 245-252.

Aggarwal, S., Ghilardi, N., Xie, M. H., de Sauvage, F. J., & Gurney, A. L. 2003, "Interleukin-23 Promotes a Distinct CD4 T Cell Activation State Characterized by the Production of Interleukin-17", *Journal of Biological Chemistry*, vol. 278, no. 3, pp. 1910-1914.

Aiuti, A., Cattaneo, F., Galimberti, S., Benninghoff, U., Cassani, B., Callegaro, L., Scaramuzza, S., Andolfi, G., Mirolo, M., Brigida, I., Tabucchi, A., Carlucci, F., Eibl, M., Aker, M., Slavin, S., Al-Mousa, H., Al Ghonaium, A., Ferster, A., Duppenthaler, A., Notarangelo, L., Wintergerst, U., Buckley, R. H., Bregni, M., Marktel, S., Valsecchi, M. G., Rossi, P., Ciceri, F., Miniero, R., Bordignon, C., & Roncarolo, M. G. 2009, "Gene Therapy for Immunodeficiency Due to Adenosine Deaminase Deficiency", *The New England Journal of Medicine*, vol. 360, no. 5, pp. 447-458.

Akeson, A. L., Wiginton, D. A., & Hutton, J. J. 1989, "Normal and mutant human adenosine deaminase genes", *J Cell Biochem.*, vol. 39, no. 3, pp. 217-228.

Ally, B. A., Hawley, T. S., Kall-Faienza, K. J., Kundig, T. M., Oehen, S. U., Pircher, H., Hawley, R. G., & Ohashi, P. S. 1995, "Prevention of autoimmune disease by retroviral-mediated gene therapy", *The Journal of Immunology*, vol. 155, no. 11, pp. 5404-5408.

Andersen, P. A., West, S. G., O'Dell, J. R., Via, C. S., Claypool, R. G., & Kotzin, B. L. 1985, "Weekly pulse methotrexate in rheumatoid arthritis. Clinical and immunologic effects in a randomized, double-blind study", *Ann.Intern.Med.*, vol. 103, no. 4, pp. 489-496.

Anderson, M. S., Venanzi, E. S., Chen, Z., Berzins, S. P., Benoist, C., & Mathis, D. 2005, "The cellular mechanism of Aire control of T cell tolerance", *Immunity*, vol. 23, no. 2, pp. 227-239.

Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von, B. H., Bronson, R., Dierich, A., Benoist, C., & Mathis, D. 2002, "Projection of an immunological self shadow within the thymus by the aire protein", *Science*, vol. 298, no. 5597, pp. 1395-1401.

Arai, K., Yamamura, S., Hanyu, T., Takahashi, H. E., Umezu, H., Watanabe, H., & Abo, T. 1996, "Extrathymic differentiation of resident T cells in the joints of mice with collagen-induced arthritis", *The Journal of Immunology*, vol. 157, no. 11, pp. 5170-5177.

Arfi, V., Lienard, J., Nguyen, X. N., Berger, G., Rigal, D., Darlix, J. L., & Cimarelli, A. 2009, "Characterization of the Behavior of Functional Viral Genomes during the Early Steps of Human Immunodeficiency Virus Type 1 Infection", *The Journal of Virology*, vol. 83, no. 15, pp. 7524-7535.

Arhel, N., Genovesio, A., Kim, K. A., Miko, S., Perret, E., Olivo-Marin, J. C., Shorte, S., & Charneau, P. 2006, "Quantitative four-dimensional tracking of cytoplasmic and nuclear HIV-1 complexes", *Nat Methods*, vol. 3, no. 10, pp. 817-824.

Arhel, N. J., Souquere-Besse, S., Munier, S., Souque, P., Guadagnini, S., Rutherford, S., Prevost, M. C., Allen, T. D., & Charneau, P. 2007, "HIV-1 DNA Flap formation promotes uncoating of the pre-integration complex at the nuclear pore", *EMBO J*, vol. 26, no. 12, pp. 3025-3037.

Arhel, N., Souquere-Besse, S., & Charneau, P. 2006, "Wild-type and central DNA flap defective HIV-1 lentiviral vector genomes: intracellular visualization at ultrastructural resolution levels", *Retrovirology*, vol. 3, no. 1, p. 38.

Arruda, L. B., Del Sim, Chikhlikar, P. R., Maciel, M., Akasaki, K., August, J. T., & Marques, E. T. A. 2006, Dendritic Cell-Lysosomal-Associated Membrane Protein (LAMP) and LAMP-1-HIV Gag Chimeras Have Distinct Cellular Trafficking Pathways and Prime T and B Cell Responses to A Diverse Repertoire of Epitopes. The Journal of Immunology . 19-5-2006.

Arumugam, P. I., Scholes, J., Perelman, N., Xia, P., Yee, J. K. and Malik, P. 2007, Improved human β-globin expression from self-inactivating lentiviral vectors carrying the chicken hypersensitive site-4 (cHS4) insulator element. Mol Ther 15: 1863–1871.

Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L., & Powrie, F. 1999, "An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation", *J Exp.Med.*, vol. 190, no. 7, pp. 995-1004.

Atkinson, M. A. & Leiter, E. H. 1999, "The NOD mouse model of type 1 diabetes: as good as it gets?", *Nat Med*, vol. 5, no. 6, pp. 601-604.

Bainbridge, J. W. B., Smith, A. J., Barker, S. S., Robbie, S., Henderson, R., Balaggan, K., Viswanathan, A., Holder, G. E., Stockman, A., Tyler, N., Petersen-Jones, S., Bhattacharya, S. S., Thrasher, A. J., Fitzke, F. W., Carter, B. J., Rubin, G. S., Moore, A. T., & Ali, R. R. 2008, "Effect of Gene Therapy on Visual Function in Leber's Congenital Amaurosis", *The New England Journal of Medicine*, vol. 358, no. 21, pp. 2231-2239.

Baltimore, D. 1970, "RNA-dependent DNA polymerase in virions of RNA tumour viruses", *Nature*, vol. 226, no. 5252, pp. 1209-1211.

Banda, N. K., Takahashi, K., Wood, A. K., Holers, V. M., & Arend, W. P. 2007, "Pathogenic Complement Activation in Collagen Antibody- Induced Arthritis in Mice Requires Amplification by the Alternative Pathway", *The Journal of Immunology*, vol. 179, no. 6, pp. 4101-4109.

Barron, L., Knoechel, B., Lohr, J., & Abbas, A. K. 2008, "Cutting Edge: Contributions of Apoptosis and Anergy to Systemic T Cell Tolerance", *The Journal of Immunology*, vol. 180, no. 5, pp. 2762-2766.

Barthlott, T., Moncrieffe, H., Veldhoen, M., Atkins, C. J., Christensen, J., O'Garra, A., & Stockinger, B. 2005, "CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production", *Int.Immunol*, vol. 17, no. 3, pp. 279-288.

Bartlett, J. S., Wilcher, R., & Samulski, R. J. 2000, "Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors", *J Virol.*, vol. 74, no. 6, pp. 2777-2785.

Batsalova, T., Vestberg, M., Holmdahl, R., & Dzhabazov, B. 2010 "MOG79-90 peptide in complex with recombinant MHC Class II molecules ameliorates experimental autoimmune encephalomyelitis", *Biotechnol. & Biotechnol. EQ.* Vol 24, no 2, pp. 107-112.

Baum, C., Hegewisch-Becker, S., Eckert, H. G., Stocking, C., & Ostertag, W. 1995, "Novel retroviral vectors for efficient expression of the multidrug resistance (mdr-1) gene in early hematopoietic cells", *J Virol.*, vol. 69, no. 12, pp. 7541-7547.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L., & Kuchroo, V. K. 2006, "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells", *Nature*, vol. 441, no. 7090, pp. 235-238.

Bielekova, B., Goodwin, B., Richert, N., Cortese, I., Kondo, T., Afshar, G., Gran, B., Eaton, J., Antel, J., Frank, J. A., McFarland, H. F., & Martin, R. 2000, "Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand", *Nat Med*, vol. 6, no. 10, pp. 1167-1175.

Billingham, R. E., Brent, L., & Medawar, P. B. 1953, "Actively acquired tolerance of foreign cells", *Nature*, vol. 172, no. 4379, pp. 603-606.

Blaese, R. M., Culver, K. W., Miller, A. D., Carter, C. S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Greenblatt, J. J., Rosenberg, S. A., Klein, H., Berger, M., Mullen, C. A., Ramsey, W. J., Muul, L., Morgan, R. A., & Anderson, W. F. 1995, "T Lymphocyte-Directed Gene Therapy for ADA$^-$ SCID: Initial Trial Results After 4 Years", *Science*, vol. 270, no. 5235, pp. 475-480.

Blobel, G. & Dobberstein, B. 1975, "Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma", *J Cell Biol.*, vol. 67, no. 3, pp. 835-851.

Bokhoven, M., Stephen, S. L., Knight, S., Gevers, E. F., Robinson, I. C., Takeuchi, Y., & Collins, M. K. 2009, "Insertional gene activation by lentiviral and gammaretroviral vectors", *J Virol.*, vol. 83, no. 1, pp. 283-294.

Bolinger, C. & Boris-Lawrie, K. 2009, "Mechanisms employed by retroviruses to exploit host factors for translational control of a complicated proteome", *Retrovirology*, vol. 6, no. 1, p. 8.

Bonehill, A., Heirman, C., & Thielemans, K. 2005, "Genetic approaches for the induction of a CD4+ T cell response in cancer immunotherapy", *J.Gene Med.*, vol. 7, no. 6, pp. 686-695.

Bonifacino, J. S. & Traub, L. M. 2003, "Signals for sorting of transmembrane proteins to endosomes and lysosomes", *Annu.Rev.Biochem.*, vol. 72, pp. 395-447.

Bopp, T., Becker, C., Klein, M., Klein-Hessling, S., Palmetshofer, A., Serfling, E., Heib, V., Becker, M., Kubach, J., Schmitt, S., Stoll, S., Schild, H., Staege, M. S., Stassen, M., Jonuleit, H., & Schmitt, E. 2007, "Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression", *J Exp.Med.*, vol. 204, no. 6, pp. 1303-1310.

Bordignon, C., Notarangelo, L. D., Nobili, N., Ferrari, G., Casorati, G., Panina, P., Mazzolari, E., Maggioni, D., Rossi, C., Servida, P., Ugazio, A. G., & Mavilio, F. 1995, "Gene Therapy in Peripheral Blood Lymphocytes and Bone Marrow for ADA-Immunodeficient Patients", *Science*, vol. 270, no. 5235, pp. 470-475.

Bouneaud, C., Kourilsky, P., & Bousso, P. 2000, "Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion", *Immunity.*, vol. 13, no. 6, pp. 829-840.

Bour-Jordan, H. & Blueston, J. A. 2002, "CD28 function: a balance of costimulatory and regulatory signals", *J.Clin.Immunol.*, vol. 22, no. 1, pp. 1-7.

Bousquet, J., Lockey, R., Malling, H. J., varez-Cuesta, E., Canonica, G. W., Chapman, M. D., Creticos, P. J., Dayer, J. M., Durham, S. R., Demoly, P., Goldstein, R. J., Ishikawa, T., Ito, K., Kraft, D., Lambert, P. H., Lowenstein, H., Muller, U., Norman, P. S., Reisman, R. E., Valenta, R., Valovirta, E., & Yssel, H. 1998, "Allergen immunotherapy: therapeutic vaccines for allergic diseases. World Health Organization. American academy of Allergy, Asthma and Immunology", *Ann Allergy Asthma Immunol*, vol. 81, no. 5 Pt 1, pp. 401-405.

Boussiotis, V. A., Freeman, G. J., Gray, G., Gribben, J., & Nadler, L. M. 1993, "B7 but not intercellular adhesion molecule-1 costimulation prevents the induction of human alloantigen-specific tolerance", *J Exp.Med*, vol. 178, no. 5, pp. 1753-1763.

Brand, D. D., Kang, A. H., & Rosloniec, E. F. 2004, "The mouse model of collagen-induced arthritis", *Methods Mol.Med.*, vol. 102, pp. 295-312.

Breckpot, K., Dullaers, M., Bonehill, A., van, M. S., Heirman, C., de, G. C., van der, B. P., & Thielemans, K. 2003, "Lentivirally transduced dendritic cells as a tool for cancer immunotherapy", *J Gene Med*, vol. 5, no. 8, pp. 654-667.

Brennan, F. M. & McInnes, I. B. 2008, "Evidence that cytokines play a role in rheumatoid arthritis", *J Clin.Invest*, vol. 118, no. 11, pp. 3537-3545.

Brown, P. O., Bowerman, B., Varmus, H. E., & Bishop, J. M. 1989, "Retroviral integration: structure of the initial covalent product and its precursor, and a role for the viral IN protein", *Proc.Natl Acad.Sci.U.S.A*, vol. 86, no. 8, pp. 2525-2529.

Brunsberg, U., Gustafsson, K., Jansson, L., Michaelsson, E., hrlund-Richter, L., Pettersson, S., Mattsson, R., & Holmdahl, R. 1994, "Expression of a transgenic class II Ab gene confers susceptibility to collagen-induced arthritis", *Eur.J Immunol*, vol. 24, no. 7, pp. 1698-1702.

Bukovsky, A. A., Dorfman, T., Weimann, A., & Gottlinger, H. G. 1997, "Nef association with human immunodeficiency virus type 1 virions and cleavage by the viral protease", *J Virol.*, vol. 71, no. 2, pp. 1013-1018.

Burke, C. J., Sanyal, G., Bruner, M. W., Ryan, J. A., LaFemina, R. L., Robbins, H. L., Zeft, A. S., Middaugh, C. R., & Cordingley, M. G. 1992, "Structural implications of spectroscopic characterization of a putative zinc finger peptide from HIV-1 integrase", *J Biol.Chem.*, vol. 267, no. 14, pp. 9639-9644.

Burkhardt, H., Koller, T., Engstrom, A., Nandakumar, K. S., Turnay, J., Kraetsch, H. G., Kalden, J. R., & Holmdahl, R. 2002, "Epitope-specific recognition of type II collagen by rheumatoid arthritis antibodies is shared with recognition by antibodies that are arthritogenic in collagen-induced arthritis in the mouse", *Arthritis Rheum.*, vol. 46, no. 9, pp. 2339-2348.

Bushman, F. D. & Craigie, R. 1991, "Activities of human immunodeficiency virus (HIV) integration protein in vitro: specific cleavage and integration of HIV DNA", *Proc.Natl Acad.Sci.U.S.A*, vol. 88, no. 4, pp. 1339-1343.

Butsch, M. & Boris-Lawrie, K. 2002, "Destiny of Unspliced Retroviral RNA: Ribosome and/or Virion?", *The Journal of Virology*, vol. 76, no. 7, pp. 3089-3094.

Camaur, D. & Trono, D. 1996, "Characterization of human immunodeficiency virus type 1 Vif particle incorporation", *J Virol.*, vol. 70, no. 9, pp. 6106-6111.

Campbell, E. M., Perez, O., Melar, M., & Hope, T. J. 2007, "Labeling HIV-1 virions with two fluorescent proteins allows identification of virions that have productively entered the target cell", *Virology*, vol. 360, no. 2, pp. 286-293.

Carrier, Y., Yuan, J., Kuchroo, V. K., & Weiner, H. L. 2007, "Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice", *The Journal of Immunology*, vol. 178, no. 1, pp. 179-185.

Casellas, R., Shih, T. A., Kleinewietfeld, M., Rakonjac, J., Nemazee, D., Rajewsky, K., & Nussenzweig, M. C. 2001, "Contribution of receptor editing to the antibody repertoire", *Science*, vol. 291, no. 5508, pp. 1541-1544.

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint, B. G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Deist, F. L., & Fischer, A. 2000, "Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease", *Science*, vol. 288, no. 5466, pp. 669-672.

Chaillous, L., Lefevre, H., Thivolet, C., Boitard, C., Lahlou, N., tlan-Gepner, C., Bouhanick, B., Mogenet, A., Nicolino, M., Carel, J. C., Lecomte, P., Marechaud, R., Bougneres, P., Charbonnel, B., & Sai, P. 2000, "Oral insulin administration and residual beta-cell function in recent-onset type 1 diabetes: a multicentre randomised controlled trial. Diabete Insuline Orale group", *Lancet*, vol. 356, no. 9229, pp. 545-549.

Charneau, P. & Clavel, F. 1991, "A single-stranded gap in human immunodeficiency virus unintegrated linear DNA defined by a central copy of the polypurine tract", *J Virol.*, vol. 65, no. 5, pp. 2415-2421.

Chen, H. & Engelman, A. 2000, "Characterization of a Replication-Defective Human Immunodeficiency Virus Type 1 att Site Mutant That Is Blocked after the 3' Processing Step of Retroviral Integration", *The Journal of Virology*, vol. 74, no. 17, pp. 8188-8193.

Chen, P. M., Chiou, T. J., Hsieh, R. K., Fan, F. S., Chu, C. J., Lin, C. Z., Chiang, H., Yen, C. C., Wang, W. S., & Liu, J. H. 1999, "p53 gene mutations and rearrangements in non-Hodgkin's lymphoma", *Cancer*, vol. 85, no. 3, pp. 718-724.

Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G., & Wahl, S. M. 2003, "Conversion of peripheral CD4+", *J Exp.Med.*, vol. 198, no. 12, pp. 1875-1886.

Chiocchia, G., Boissier, M. C., & Fournier, C. 1991, "Therapy against murine collagen-induced arthritis with T cell receptor V beta-specific antibodies", *Eur.J.Immunol.*, vol. 21, no. 12, pp. 2899-2905.

Clarke, J. D. & Jayasinghe, S. N. 2008, "Bio-electrosprayed multicellular zebrafish embryos are viable and develop normally", *Biomed.Mater.*, vol. 3, no. 1, p. 11001.

Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., Cross, R., Sehy, D., Blumberg, R. S., & Vignali, D. A. 2007, "The inhibitory cytokine IL-35 contributes to regulatory T-cell function", *Nature*, vol. 450, no. 7169, pp. 566-569.

Collison, L. W., Pillai, M. R., Chaturvedi, V., & Vignali, D. A. A. 2009, "Regulatory T Cell Suppression Is Potentiated by Target T Cells in a Cell Contact, IL-35- and IL-10-Dependent Manner", *The Journal of Immunology*, vol. 182, no. 10, pp. 6121-6128.

Cornall, R. J., Goodnow, C. C., & Cyster, J. G. 1999, "Regulation of B cell antigen receptor signaling by the Lyn/CD22/SHP1 pathway", *Curr.Top.Microbiol.Immunol.*, vol. 244, pp. 57-68.

Corthay, A., Backlund, J., Broddefalk, J., Michaelsson, E., Goldschmidt, T. J., Kihlberg, J., & Holmdahl, R. 1998, "Epitope glycosylation plays a critical role for T cell recognition of type II collagen in collagen-induced arthritis", *Eur.J Immunol*, vol. 28, no. 8, pp. 2580-2590.

Corthay, A., Backlund, J., & Holmdahl, R. 2001, "Role of glycopeptide-specific T cells in collagen-induced arthritis: an example how post-translational modification of proteins may be involved in autoimmune disease", *Ann.Med.*, vol. 33, no. 7, pp. 456-465.

Corthay, A., Johansson, A., Vestberg, M., & Holmdahl, R. 1999, "Collagen-induced arthritis development requires alpha beta T cells but not gamma delta T cells: studies with T cell-deficient (TCR mutant) mice", *Int.Immunol*, vol. 11, no. 7, pp. 1065-1073.

Courtenay, J. S., Dallman, M. J., Dayan, A. D., Martin, A., & Mosedale, B. 1980, "Immunisation against heterologous type II collagen induces arthritis in mice", *Nature*, vol. 283, pp. 666-668.

Courties, G., Presumey, J., Duroux-Richard, I., Jorgensen, C., & Apparailly, F. 2009, "RNA interference-based gene therapy for successful treatment of rheumatoid arthritis", *Expert Opinion on Biological Therapy*, vol. 9, no. 5, p. 535.

Cronin, J., Zhang, X. Y., & Reiser, J. 2005, "Altering the tropism of lentiviral vectors through pseudotyping", *Curr.Gene Ther.*, vol. 5, pp. 387-398.

Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S. A., Gorman, D., Kastelein, R. A., & Sedgwick, J. D. 2003, "Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain", *Nature*, vol. 421, no. 6924, pp. 744-748.

Cutolo, M., Sulli, A., Pizzorni, C., Seriolo, B., & Straub, R. H. 2001, "Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis", *Ann.Rheum.Dis.*, vol. 60, no. 8, pp. 729-735.

Dalgleish, A. G., Beverley, P. C., Clapham, P. R., Crawford, D. H., Greaves, M. F., & Weiss, R. A. 1984, "The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus", *Nature*, vol. 312, no. 5996, pp. 763-767.

Danos, O. & Mulligan, R. C. 1988, "Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges", *Proc.Natl Acad.Sci.U.S.A*, vol. 85, no. 17, pp. 6460-6464.

Darlix, J. L., Gabus, C., Nugeyre, M. T., Clavel, F., & Barre-Sinoussi, F. 1990, "Cis elements and trans-acting factors involved in the RNA dimerization of the human immunodeficiency virus HIV-1", *J Mol.Biol.*, vol. 216, no. 3, pp. 689-699.

de la Rosa. M., Rutz, S., Dorninger, H., & Scheffold, A. 2004, "Interleukin-2 is essential for CD4+CD25+ regulatory T cell function", *Eur.J Immunol*, vol. 34, no. 9, pp. 2480-2488.

de Noronha, C. M., Sherman, M. P., Lin, H. W., Cavrois, M. V., Moir, R. D., Goldman, R. D., & Greene, W. C. 2001, "Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr", *Science*, vol. 294, no. 5544, pp. 1105-1108.

Dhodapkar, M. V., Steinman, R. M., Krasovsky, J., Munz, C., & Bhardwaj, N. 2001, "Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells", *J Exp.Med*, vol. 193, no. 2, pp. 233-238.

Dieckmann, D., Plottner, H., Berchtold, S., Berger, T., & Schuler, G. 2001, "Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood", *J Exp.Med.*, vol. 193, no. 11, pp. 1303-1310.

Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., & Paxton, W. A. 1996, "HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5", *Nature*, vol. 381, no. 6584, pp. 667-673.

Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., & Naldini, L. 1998, "A third-generation lentivirus vector with a conditional packaging system", *J Virol.*, vol. 72, no. 11, pp. 8463-8471.

Dzhambazov, B., Nandakumar, K. S., Kihlberg, J., Fugger, L., Holmdahl, R., & Vestberg, M. 2006, "Therapeutic vaccination of active arthritis with a glycosylated collagen type II peptide in complex with MHC class II molecules", *The Journal of Immunology*, vol. 176, no. 3, pp. 1525-1533.

Eagles, P. A., Qureshi, A. N., & Jayasinghe, S. N. 2006, "Electrohydrodynamic jetting of mouse neuronal cells", *Biochem.J.*, vol. 394, no. Pt 2, pp. 375-378.

Edwards, R. A. & Rohwer, F. 2005, "Viral metagenomics", *Nat Rev Microbiol.*, vol. 3, no. 6, pp. 504-510.

Ehinger, M., Vestberg, M., Johansson, A. C., Johannesson, M., Svensson, A., & Holmdahl, R. 2001, "Influence of CD4 or CD8 deficiency on collagen-induced arthritis", *Immunology*, vol. 103, no. 3, pp. 291-300.

Eixarch, H., Espejo, C., Gomez, A., Mansilla, M. J., Castillo, M., Mildner, A., Vidal, F., Gimeno, R., Prinz, M., Montalban, X., & Barquinero, J. 2009, "Tolerance induction in experimental autoimmune encephalomyelitis using non-myeloablative hematopoietic gene therapy with autoantigen", *Mol.Ther*, vol. 17, no. 5, pp. 897-905.

Ellison, V., Gerton, J., Vincent, K. A., & Brown, P. O. 1995, "An Essential Interaction between Distinct Domains of HIV-1 Integrase Mediates Assembly of the Active Multimer", *Journal of Biological Chemistry*, vol. 270, no. 7, pp. 3320-3326.

Ernst, R. K., Bray, M., Rekosh, D., & Hammarskjold, M. L. 1997, "A structured retroviral RNA element that mediates nucleocytoplasmic export of intron-containing RNA", *Molecular and Cellular Biology*, vol. 17, no. 1, pp. 135-144.

Esslinger, C., Romero, P., & MacDonald, H. R. 2002, "Efficient transduction of dendritic cells and induction of a T-cell response by third-generation lentivectors", *Hum.Gene Ther*, vol. 13, no. 9, pp. 1091-1100.

Evans, C. H., Ghivizzani, S. C., & Robbins, P. D. 2009, "Gene therapy of the rheumatic diseases: 1998 to 2008", *Arthritis Res.Ther*, vol. 11, no. 1, p. 209.

Evans, C. H., Ghivizzani, S. C., & Robbins, P. D. 2006, "Gene therapy for arthritis: what next?", *Arthritis Rheum.*, vol. 54, no. 6, pp. 1714-1729.

Evans, C. H., Ghivizzani, S. C., & Robbins, P. D. 2008, "Arthritis gene therapy's first death", *Arthritis Res.Ther*, vol. 10, no. 3, p. 110.

Evans, C. H., Robbins, P. D., Ghivizzani, S. C., Herndon, J. H., Kang, R., Bahnson, A. B., Barranger, J. A., Elders, E. M., Gay, S., Tomaino, M. M., Wasko, M. C., Watkins, S. C., Whiteside, T. L., Glorioso, J. C., Lotze, M. T., & Wright, T. M. 1996, "Clinical trial to assess the safety, feasibility, and efficacy of transferring a potentially anti-arthritic cytokine gene to human joints with rheumatoid arthritis", *Hum.Gene Ther*, vol. 7, no. 10, pp. 1261-1280.

Evans, C. H., Robbins, P. D., Ghivizzani, S. C., Wasko, M. C., Tomaino, M. M., Kang, R., Muzzonigro, T. A., Vogt, M., Elder, E. M., Whiteside, T. L., Watkins, S. C., & Herndon, J. H. 2005, "Gene transfer to human joints: progress toward a gene therapy of arthritis", *Proc.Natl Acad.Sci.U.S.A*, vol. 102, no. 24, pp. 8698-8703.

Fantini, M. C., Becker, C., Monteleone, G., Pallone, F., Galle, P. R., & Neurath, M. F. 2004, "Cutting edge: TGF-beta induces a regulatory phenotype in CD4+", *The Journal of Immunology*, vol. 172, no. 9, pp. 5149-5153.

Fassati, A. & Goff, S. P. 1999, "Characterization of intracellular reverse transcription complexes of Moloney murine leukemia virus", *J Virol.*, vol. 73, no. 11, pp. 8919-8925.

Felice, B., Cattoglio, C., Cittaro, D., Testa, A., Miccio, A., Ferrari, G., Luzi, L., Recchia, A., & Mavilio, F. 2009, "Transcription factor binding sites are genetic determinants of retroviral integration in the human genome", *PLoS.One.*, vol. 4, no. 2, p. e4571.

Feng, Y., Broder, C. C., Kennedy, P. E., & Berger, E. A. 1996, "HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor", *Science*, vol. 272, no. 5263, pp. 872-877.

Fleischmann, R. M., Tesser, J., Schiff, M. H., Schechtman, J., Burmester, G. R., Bennett, R., Modafferi, D., Zhou, L., Bell, D., & Appleton, B. 2006, "Safety of extended treatment with anakinra in patients with rheumatoid arthritis", *Annals of the Rheumatic Diseases*, vol. 65, no. 8, pp. 1006-1012.

Fornerod, M., Ohno, M., Yoshida, M., & Mattaj, I. W. 1997, "CRM1 is an export receptor for leucine-rich nuclear export signals", *Cell*, vol. 90, no. 6, pp. 1051-1060.

Fowlkes, B. J. & Schweighoffer, E. 1995, "Positive selection of T cells", *Curr.Opin.Immunol.*, vol. 7, no. 2, pp. 188-195.

Franco, A., Southwood, S., Arrhenius, T., Kuchroo, V. K., Grey, H. M., Sette, A., & Ishioka, G. Y. 1994, "T cell receptor antagonist peptides are highly effective inhibitors of experimental allergic encephalomyelitis", *Eur.J Immunol*, vol. 24, no. 4, pp. 940-946.

Frank, K. M., Hogarth, D. K., Miller, J. L., Mandal, S., Mease, P. J., Samulski, R. J., Weisgerber, G. A., & Hart, J. 2009, "Investigation of the Cause of Death in a Gene-Therapy Trial", *The New England Journal of Medicine*, vol. 361, no. 2, pp. 161-169.

Fujimoto, M., Serada, S., Mihara, M., Uchiyama, Y., Yoshida, H., Koike, N., Ohsugi, Y., Nishikawa, T., Ripley, B., Kimura, A., Kishimoto, T., & Naka, T. 2008, "Interleukin-6 blockade suppresses autoimmune arthritis in mice by the inhibition of inflammatory Th17 responses", *Arthritis Rheum.*, vol. 58, no. 12, pp. 3710-3719.

Fukuda, M. 1991, "Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking", *J Biol.Chem.*, vol. 266, no. 32, pp. 21327-21330.

Ganan-Calvo, A. M. & Gordillo, J. M. 2001, "Perfectly monodisperse microbubbling by capillary flow focusing", *Phys.Rev.Lett.*, vol. 87, no. 27 Pt 1, p. 274501.

Ganser-Pornillos, B. K., Yeager, M., & Sundquist, W. I. 2008, "The structural biology of HIV assembly", *Current Opinion in Structural Biology*, vol. 18, no. 2, pp. 203-217.

Gaspar, H. B., Bjorkegren, E., Parsley, K., Gilmour, K. C., King, D., Sinclair, J., Zhang, F., Giannakopoulos, A., Adams, S., Fairbanks, L. D., Gaspar, J., Henderson, L., Xu-Bayford, J. H., Davies, E. G., Veys, P. A., Kinnon, C., & Thrasher, A. J. 2006, "Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning", *Mol.Ther*, vol. 14, no. 4, pp. 505-513.

Gaspar, H. B., Parsley, K. L., Howe, S., King, D., Gilmour, K. C., Sinclair, J., Brouns, G., Schmidt, M., Von, K. C., Barington, T., Jakobsen, M. A., Christensen, H. O., Al, G. A., White, H. N., Smith, J. L., Levinsky, R. J., Ali, R. R., Kinnon, C., & Thrasher, A. J. 2004, "Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector", *Lancet*, vol. 364, no. 9452, pp. 2181-2187.

Gaudreau, S., Guindi, C., Menard, M., Besin, G., Dupuis, G., & Amrani, A. 2007, "Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of CD4+CD25+ regulatory T cells", *J Immunol*, vol. 179, no. 6, pp. 3638-3647.

Gaur, A., Wiers, B., Liu, A., Rothbard, J., & Fathman, C. G. 1992, "Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy", *Science*, vol. 258, no. 5087, pp. 1491-1494.

Geach, T. J., Mongkoldhumrongkul, N., Zimmerman, L. B., & Jayasinghe, S. N. 2009, "Bio-electrospraying living Xenopus tropicalis embryos: investigating the structural, functional and biological integrity of a model organism", *Analyst*, vol. 134, no. 4, pp. 743-747.

Gerards, A. H., de Lathouder, S., de Groot, E. R., Dijkmans, B. A. C., & Aarden, L. A. 2003, "Inhibition of cytokine production by methotrexate. Studies in healthy volunteers and patients with rheumatoid arthritis", *Rheumatology*, vol. 42, no. 10, pp. 1189-1196.

Ghivizzani, S. C., Lechman, E. R., Kang, R., Tio, C., Kolls, J., Evans, C. H., & Robbins, P. D. 1998, "Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to rabbit knees with experimental

arthritis has local and distal anti-arthritic effects", *Proc.Natl Acad.Sci.U.S.A*, vol. 95, no. 8, pp. 4613-4618.

Gianni, A. M., Smotkin, D., & Weinberg, R. A. 1975, "Murine leukemia virus: detection of unintegrated double-stranded DNA forms of the provirus", *Proc.Natl Acad.Sci.U.S.A*, vol. 72, no. 2, pp. 447-451.

Gilliet, M. & Liu, Y. J. 2002, "Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells", *J Exp.Med*, vol. 195, no. 6, pp. 695-704.

Gjertsson, I., Laurie, K. L., Devitt, J., Howe, S. J., Thrasher, A. J., Holmdahl, R., & Gustafsson, K. 2009, "Tolerance induction using lentiviral gene delivery delays onset and severity of collagen II arthritis", *Mol.Ther*, vol. 17, no. 4, pp. 632-640.

Goodnow, C. C., Crosbie, J., Adelstein, S., Lavoie, T. B., Smith-Gill, S. J., Brink, R. A., Pritchard-Briscoe, H., Wotherspoon, J. S., Loblay, R. H., Raphael, K., & . 1988, "Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice", *Nature*, vol. 334, no. 6184, pp. 676-682.

Goodnow, C. C., Sprent, J., Fazekas de St, G. B., & Vinuesa, C. G. 2005, "Cellular and genetic mechanisms of self tolerance and autoimmunity", *Nature*, vol. 435, no. 7042, pp. 590-597.

Gouze, E., Pawliuk, R., Gouze, J. N., Pilapil, C., Fleet, C., Palmer, G. D., Evans, C. H., Leboulch, P., & Ghivizzani, S. C. 2003, "Lentiviral-mediated gene delivery to synovium: potent intra-articular expression with amplification by inflammation", *Mol.Ther*, vol. 7, no. 4, pp. 460-466.

Gravallese, E. M. 2002, "Bone destruction in arthritis", *Ann.Rheum.Dis.*, vol. 61 Suppl 2, p. ii84-ii86.

Grieger, J. C., Choi, V. W., & Samulski, R. J. 2006, "Production and characterization of adeno-associated viral vectors", *Nat Protoc.*, vol. 1, no. 3, pp. 1412-1428.

Guangyu Ma, Hideaki Shimada, Kenzo Hiroshima, Yuji Tada, Nobuo Suzuki, & Masatoshi Tagawa 2008, "Gene medicine for cancer treatment:Commercially available medicine and accumulated clinical data in China", *Drug Design, Development and Therapy* pp. 115-122.

Gustafsson, K., Karlsson, M., Andersson, L., & Holmdahl, R. 1990, "Structures on the I-A molecule predisposing for susceptibility to type II collagen-induced autoimmune arthritis", *Eur.J Immunol*, vol. 20, no. 9, pp. 2127-2131.

Hacein-Bey-Abina, S., Garrigue, A., Wang, G. P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., Asnafi, V., MacIntyre, E., Dal, C. L., Radford, I., Brousse, N., Sigaux, F., Moshous, D., Hauer, J., Borkhardt, A., Belohradsky, B. H., Wintergerst, U., Velez, M. C., Leiva, L., Sorensen, R., Wulffraat, N., Blanche, S., Bushman, F. D., Fischer, A., & Cavazzana-Calvo, M. 2008, "Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1", *J Clin.Invest*, vol. 118, no. 9, pp. 3132-3142.

Hacein-Bey-Abina, S., le, D. F., Carlier, F., Bouneaud, C., Hue, C., De Villartay, J. P., Thrasher, A. J., Wulffraat, N., Sorensen, R., Dupuis-Girod, S., Fischer, A., Davies, E. G., Kuis, W., Leiva, L., & Cavazzana-Calvo, M. 2002, "Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy", *N Engl J Med*, vol. 346, no. 16, pp. 1185-1193.

Halverson, R., Torres, R. M., & Pelanda, R. 2004, "Receptor editing is the main mechanism of B cell tolerance toward membrane antigens", *Nat.Immunol.*, vol. 5, no. 6, pp. 645-650.

Hara, M., Kingsley, C. I., Niimi, M., Read, S., Turvey, S. E., Bushell, A. R., Morris, P. J., Powrie, F., & Wood, K. J. 2001, "IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo", *The Journal of Immunology*, vol. 166, no. 6, pp. 3789-3796.

Hargrove, P. W., Kepes, S., Hanawa, H., Obenauer, J. C., Pei, D., Cheng, C., Gray, J. T., Neale, G., & Persons, D. A. 2008, "Globin lentiviral vector insertions can perturb the expression of endogenous genes in beta-thalassemic hematopoietic cells", *Mol.Ther*, vol. 16, no. 3, pp. 525-533.

Harris, M. E. & Hope, T. J. 2000, "RNA export: insights from viral models", *Essays Biochem.*, vol. 36, pp. 115-127.

Heikkinen, J., Risteli, M., Wang, C., Latvala, J., Rossi, M., Valtavaara, M., & Myllyla, R. 2000, "Lysyl hydroxylase 3 is a multifunctional protein possessing collagen glucosyltransferase activity", *J Biol.Chem.*, vol. 275, no. 46, pp. 36158-36163.

Hegde, R. S. & Kang, S. W. 2008, "The concept of translocational regulation", *J Cell Biol.*, vol. 182, no. 2, pp. 225-232.

Hegen, M., Gaestel, M., Nickerson-Nutter, C. L., Lin, L. L., & Telliez, J. B. 2006, "MAPKAP kinase 2-deficient mice are resistant to collagen-induced arthritis", *The Journal of Immunology*, vol. 177, no. 3, pp. 1913-1917.

Hildeman, D. A., Zhu, Y., Mitchell, T. C., Kappler, J., & Marrack, P. 2002, "Molecular mechanisms of activated T cell death in vivo", *Curr.Opin.Immunol*, vol. 14, no. 3, pp. 354-359.

Hippen, K. L., Schram, B. R., Tze, L. E., Pape, K. A., Jenkins, M. K., & Behrens, T. W. 2005, "In vivo assessment of the relative contributions of deletion, anergy, and editing to B cell self-tolerance", *The Journal of Immunology*, vol. 175, no. 2, pp. 909-916.

Ho, P. P., Higgins, J. P., Kidd, B. A., Tomooka, B., Digennaro, C., Lee, L. Y., de Vegvar, H. E., Steinman, L., & Robinson, W. H. 2006, "Tolerizing DNA vaccines for autoimmune arthritis", *Autoimmunity*, vol. 39, no. 8, pp. 675-682.

Holmdahl, R., Jansson, L., Gullberg, D., Rubin, K., Forsberg, P. O., & Klareskog, L. 1985a, "Incidence of arthritis and autoreactivity of anti-collagen antibodies after immunization of DBA/1 mice with heterologous and autologous collagen II", *Clin.Exp.Immunol.*, vol. 62, no. 3, pp. 639-646.

Holmdahl, R., Jansson, L., Larsson, A., & Jonsson, R. 1990, "Arthritis in DBA/1 mice induced with passively transferred type II collagen immune serum. Immunohistopathology and serum levels of anti-type II collagen auto-antibodies", *Scand.J Immunol*, vol. 31, no. 2, pp. 147-157.

Holmdahl, R., Jansson, L., Larsson, E., Rubin, K., & Klareskog, L. 1986, "Homologous type II collagen induces chronic and progressive arthritis in mice", *Arthritis Rheum.*, vol. 29, no. 1, pp. 106-113.

Holmdahl, R., Jonsson, R., Larsson, P., & Klareskog, L. 1988, "Early appearance of activated CD4+ T lymphocytes and class II antigen-expressing cells in joints of DBA/1 mice immunized with type II collagen", *Lab Invest*, vol. 58, no. 1, pp. 53-60.

Holmdahl, R., Klareskog, L., Rubin, K., Larsson, E., & Wigzell, H. 1985b, "T lymphocytes in collagen II-induced arthritis in mice. Characterization of arthritogenic collagen II-specific T-cell lines and clones", *Scand.J.Immunol.*, vol. 22, no. 3, pp. 295-306.

Holmdahl, R., Tarkowski, A., & Jonsson, R. 1991, "Involvement of macrophages and dendritic cells in synovial inflammation of collagen induced arthritis in DBA/1 mice and spontaneous arthritis in MRL/lpr mice", *Autoimmunity*, vol. 8, no. 4, pp. 271-280.

Hori, S., Nomura, T., & Sakaguchi, S. 2003, "Control of regulatory T cell development by the transcription factor Foxp3", *Science*, vol. 299, no. 5609, pp. 1057-1061.

Howe, S. J., Mansour, M. R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M. H., Pike-Overzet, K., Chatters, S. J., de, R. D., Gilmour, K. C., Adams, S., Thornhill, S. I., Parsley, K. L., Staal, F. J., Gale, R. E., Linch, D. C., Bayford, J., Brown, L., Quaye, M., Kinnon, C., Ancliff, P., Webb, D. K., Schmidt, M., Von, K. C., Gaspar, H. B., & Thrasher, A. J. 2008, "Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients", *J Clin.Invest*, vol. 118, no. 9, pp. 3143-3150.

Hurlbert, J. C. & Izard, T. 2002, "Crystallization of HLA-DR4 fused to an immunodominant collagen II peptide implicated in rheumatoid arthritis", *Acta Crystallogr.D.Biol.Crystallogr.*, vol. 58, no. Pt 10 Pt 1, pp. 1749-1751.

Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F., & Sakaguchi, S. 1999, "Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance", *J.Immunol.*, vol. 162, no. 9, pp. 5317-5326.

Ivanov, I. I., McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J., & Littman, D. R. 2006, "The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells", *Cell*, vol. 126, no. 6, pp. 1121-1133.

Jacks, T. & Varmus, H. E. 1985, "Expression of the Rous sarcoma virus pol gene by ribosomal frameshifting", *Science*, vol. 230, no. 4731, pp. 1237-1242.

Jaeckel, E., Kretschmer, K., Apostolou, I., & von Boehmer, H. 2006, "Instruction of Treg commitment in peripheral T cells is suited to reverse autoimmunity", *Seminars in Immunology*, vol. 18, no. 2, pp. 89-92.

Jaskolski, M., Alexandratos, J. N., Bujacz, G., & Wlodawer, A. 2009, "Piecing together the structure of retroviral integrase, an important target in AIDS therapy", *FEBS J*, vol. 276, no. 11, pp. 2926-2946.

Jayasinghe, S. N. 2007, "Bio-electrosprays: the development of a promising tool for regenerative and therapeutic medicine", *Biotechnol.J.*, vol. 2, no. 8, pp. 934-937.

Jiang, M., Mak, J., Ladha, A., Cohen, E., Klein, M., Rovinski, B., & Kleiman, L. 1993, "Identification of tRNAs incorporated into wild-type and mutant human immunodeficiency virus type 1", *J Virol.*, vol. 67, no. 6, pp. 3246-3253.

Joe, B. & Wilder, R. L. 1999, "Animal models of rheumatoid arthritis", *Mol.Med.Today*, vol. 5, no. 8, pp. 367-369.

Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A., Knop, J., & Enk, A. H. 2001, "Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood", *J Exp.Med.*, vol. 193, no. 11, pp. 1285-1294.

Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Holenbeck, A. E., Lerman, M. A., Naji, A., & Caton, A. J. 2001, "Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide", *Nat Immunol*, vol. 2, no. 4, pp. 301-306.

Ju, G. & Skalka, A. M. 1980, "Nucleotide sequence analysis of the long terminal repeat (LTR) of avian retroviruses: structural similarities with transposable elements", *Cell*, vol. 22, no. 2 Pt 2, pp. 379-386.

Kabelitz, D., Pohl, T., & Pechhold, K. 1993, "Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes", *Immunol Today*, vol. 14, no. 7, pp. 338-339.

Kaiser, J. 2007, "Clinical research. Death prompts a review of gene therapy vector", *Science*, vol. 317, no. 5838, p. 580.

Kakimoto, K., Katsuki, M., Hirofuji, T., Iwata, H., & Koga, T. 1988, "Isolation of T cell line capable of protecting mice against collagen-induced arthritis", *J.Immunol.*, vol. 140, no. 1, pp. 78-83.

Kang, Y., Melo, M., Deng, E., Tisch, R., el-Amine, M., & Scott, D. W. 1999, "Induction of hyporesponsiveness to intact foreign protein via retroviral-mediated gene expression: the IgG scaffold is important for induction and maintenance of immune hyporesponsiveness", *Proc.Natl Acad.Sci.U.S.A*, vol. 96, no. 15, pp. 8609-8614.

Kappler, J. W., Roehm, N., & Marrack, P. 1987, "T cell tolerance by clonal elimination in the thymus", *Cell*, vol. 49, no. 2, pp. 273-280.

Karwacz, K., Mukherjee, S., Apolonia, L., Blundell, M. P., Bouma, G., Escors, D., Collins, M. K., & Thrasher, A. J. 2009, "Nonintegrating lentivector vaccines stimulate prolonged T-cell and antibody responses and are effective in tumor therapy", *J Virol.*, vol. 83, no. 7, pp. 3094-3103.

Katzman, M., Katz, R. A., Skalka, A. M., & Leis, J. 1989, "The avian retroviral integration protein cleaves the terminal sequences of linear viral DNA at the in vivo sites of integration", *J Virol.*, vol. 63, no. 12, pp. 5319-5327.

Kay, J. D., Gouze, E., Oligino, T. J., Gouze, J. N., Watson, R. S., Levings, P. P., Bush, M. L., Dacanay, A., Nickerson, D. M., Robbins, P. D., Evans, C. H., & Ghivizzani, S. C. 2009, "Intra-articular gene delivery and expression of

interleukin-1Ra mediated by self-complementary adeno-associated virus", *J Gene Med*, vol. 11, no. 7, pp. 605-614.

Khoury, S. J., Lider, O., al-Sabbagh, A., & Weiner, H. L. 1990, "Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. III. Synergistic effect of lipopolysaccharide", *Cell Immunol*, vol. 131, no. 2, pp. 302-310.

Kingsley, C. I., Karim, M., Bushell, A. R., & Wood, K. J. 2002, "CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses", *The Journal of Immunology*, vol. 168, no. 3, pp. 1080-1086.

Kino, T., Gragerov, A., Slobodskaya, O., Tsopanomichalou, M., Chrousos, G. P., & Pavlakis, G. N. 2002, "Human Immunodeficiency Virus Type 1 (HIV-1) Accessory Protein Vpr Induces Transcription of the HIV-1 and Glucocorticoid-Responsive Promoters by Binding Directly to p300/CBP Coactivators", *The Journal of Virology*, vol. 76, no. 19, pp. 9724-9734.

Kishimoto, H. & Sprent, J. 1997, "Negative selection in the thymus includes semimature T cells", *The Journal of Experimental Medicine*, vol. 185, no. 2, pp. 263-271.

Kisielow, P., Bluthmann, H., Staerz, U. D., Steinmetz, M., & von, B. H. 1988, "Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes", *Nature*, vol. 333, no. 6175, pp. 742-746.

Kjellen, P., Brunsberg, U., Broddefalk, J., Hansen, B., Vestberg, M., Ivarsson, I., Engstrom, A., Svejgaard, A., Kihlberg, J., Fugger, L., & Holmdahl, R. 1998, "The structural basis of MHC control of collagen-induced arthritis; binding of the immunodominant type II collagen 256-270 glycopeptide to H-2Aq and H-2Ap molecules", *Eur.J Immunol*, vol. 28, no. 2, pp. 755-767.

Kohn, D. B., Sadelain, M., & Glorioso, J. C. 2003, "Occurrence of leukaemia following gene therapy of X-linked SCID", *Nat Rev Cancer*, vol. 3, no. 7, pp. 477-488.

Kohn, D. B., Hershfield, M. S., Carbonaro, D., Shigeoka, A., Brooks, J., Smogorzewska, E. M., Barsky, L. W., Chan, R., Burotto, F., Annett, G., Nolta, J. A., Crooks, G., Kapoor, N., Eldetr, M., Wara, D., Bowen, T., Madsen, E., Synder, F. F., Bastian, J., Muul, L., Blaese, R. M., Weinberg, K., & Parkman, R. 1998, "T lymphocytes with a normal ADA gene accumulate after transplantation of transduced autologous umbilical cord blood CD34+ cells in ADA-deficient SCID neonates", *Nat Med*, vol. 4, no. 7, pp. 775-780.

Krammer, P. H., Arnold, R., & Lavrik, I. N. 2007, "Life and death in peripheral T cells", *Nat Rev Immunol*, vol. 7, no. 7, pp. 532-542.

Kremer, J. M. 1994, "The mechanism of action of methotrexate in rheumatoid arthritis: the search continues", *J Rheumatol.*, vol. 21, no. 1, pp. 1-5.

Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M. C., & von, B. H. 2005a, "Inducing and expanding regulatory T cell populations by foreign antigen", *Nat Immunol*, vol. 6, no. 12, pp. 1219-1227.

Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M. C., & von Boehmer, H. 2005b, "Inducing and expanding regulatory T cell populations by foreign antigen", *Nat Immunol*, vol. 6, no. 12, pp. 1219-1227.

Kronenberg, M. & Rudensky, A. 2005, "Regulation of immunity by self-reactive T cells", *Nature*, vol. 435, no. 7042, pp. 598-604.

La, C. A. 2009, "Natural Tregs and autoimmunity", *Front Biosci.*, vol. 14, pp. 333-343.

Latham, K. A., Whittington, K. B., Zhou, R., Qian, Z., & Rosloniec, E. F. 2005, "Ex Vivo Characterization of the Autoimmune T Cell Response in the HLA-DR1 Mouse Model of Collagen-Induced Arthritis Reveals Long-Term Activation of Type II Collagen-Specific Cells and Their Presence in Arthritic Joints", *The Journal of Immunology*, vol. 174, no. 7, pp. 3978-3985.

Lau, A. W. T., Biester, S., Cornall, R. J., & Forrester, J. V. 2008, "Lipopolysaccharide-Activated IL-10-Secreting Dendritic Cells Suppress Experimental Autoimmune Uveoretinitis by MHCII-Dependent Activation of CD62L-Expressing Regulatory T Cells", *The Journal of Immunology*, vol. 180, no. 6, pp. 3889-3899.

Lechler, R., Chai, J. G., Marelli-Berg, F., & Lombardi, G. 2001, "The contributions of T-cell anergy to peripheral T-cell tolerance", *Immunology*, vol. 103, no. 3, pp. 262-269.

Levine, S., Sowinski, R., Gruenewald, R., & Kies, M. W. 1972, "Experimental allergic encephalomyelitis. Production by myelin basic protein adsorbed on particulate adjuvants", *Immunology*, vol. 23, no. 4, pp. 609-614.

Levings, M. K., Gregori, S., Tresoldi, E., Cazzaniga, S., Bonini, C., & Roncarolo, M. G. 2005, "Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells", *Blood*, vol. 105, no. 3, pp. 1162-1169.

Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K., & Flavell, R. A. 2006, "Transforming growth factor-beta regulation of immune responses", *Annu.Rev Immunol*, vol. 24, pp. 99-146.

Liang, S., Alard, P., Zhao, Y., Parnell, S., Clark, S. L., & Kosiewicz, M. M. 2005, "Conversion of CD4+ CD25- cells into CD4+ CD25+ regulatory T cells in vivo requires B7 costimulation, but not the thymus", *The Journal of Experimental Medicine*, vol. 201, no. 1, pp. 127-137.

Liu, Z., Xu, X., Hsu, H. C., Tousson, A., Yang, P. A., Wu, Q., Liu, C., Yu, S., Zhang, H. G., & Mountz, J. D. 2003, "CII-DC-AdTRAIL cell gene therapy inhibits infiltration of CII-reactive T cells and CII-induced arthritis", *J Clin.Invest*, vol. 112, no. 9, pp. 1332-1341.

Lo, D., Burkly, L. C., Flavell, R. A., Palmiter, R. D., & Brinster, R. L. 1989, "Tolerance to class II MHC in transgenic mice", *Semin.Immunol.*, vol. 1, no. 2, pp. 147-153.

Lu, Y. L., Spearman, P., & Ratner, L. 1993, "Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions", *J Virol.*, vol. 67, no. 11, pp. 6542-6550.

Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., & Goff, S. P. 1993, "Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B", *Cell*, vol. 73, no. 6, pp. 1067-1078.

Lubberts, E. 2003, "The role of IL-17 and family members in the pathogenesis of arthritis", *Curr.Opin.Investig.Drugs*, vol. 4, no. 5, pp. 572-577.

Mackler, B. F. 1972, "Effect of concanavalin A on human lymphoid cell lines and normal peripheral lymphocytes", *J.Natl.Cancer Inst.*, vol. 49, no. 4, pp. 935-941.

Maguire, A. M., Simonelli, F., Pierce, E. A., Pugh, E. N., Jr., Mingozzi, F., Bennicelli, J., Banfi, S., Marshall, K. A., Testa, F., Surace, E. M., Rossi, S., Lyubarsky, A., Arruda, V. R., Konkle, B., Stone, E., Sun, J., Jacobs, J., Dell'Osso, L., Hertle, R., Ma, J. x., Redmond, T. M., Zhu, X., Hauck, B., Zelenaia, O., Shindler, K. S., Maguire, M. G., Wright, J. F., Volpe, N. J., McDonnell, J. W., Auricchio, A., High, K. A., & Bennett, J. 2008, "Safety and Efficacy of Gene Transfer for Leber's Congenital Amaurosis", *The New England Journal of Medicine*, vol. 358, no. 21, pp. 2240-2248.

Mahnke, K., Qian, Y., Knop, J., & Enk, A. H. 2003, "Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells", *Blood*, vol. 101, no. 12, pp. 4862-4869.

Maksimow, M., Miiluniemi, M., Marttila-Ichihara, F., Jalkanen, S., & Hanninen, A. 2006, "Antigen targeting to endosomal pathway in dendritic cell vaccination activates regulatory T cells and attenuates tumor immunity", *Blood*, vol. 108, no. 4, pp. 1298-1305.

Malmstrom, V., Kjellen, P., & Holmdahl, R. 1998, "Type II collagen in cartilage evokes peptide-specific tolerance and skews the immune response", *J.Autoimmun.*, vol. 11, no. 3, pp. 213-221.

Malmstrom, V., Michaelsson, E., Burkhardt, H., Mattsson, R., Vuorio, E., & Holmdahl, R. 1996, "Systemic versus cartilage-specific expression of a type II collagen-specific T-cell epitope determines the level of tolerance and susceptibility to arthritis", *Proc.Natl Acad.Sci.U.S.A*, vol. 93, no. 9, pp. 4480-4485.

Malmstrom, V., Trollmo, C., & Klareskog, L. 2004, "The additive role of innate and adaptive immunity in the development of arthritis", *Am.J Med.Sci.*, vol. 327, no. 4, pp. 196-201.

Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R., & Weaver, C. T. 2006, "Transforming growth factor-beta induces development of the T(H)17 lineage", *Nature*, vol. 441, no. 7090, pp. 231-234.

Mann, R., Mulligan, R. C., & Baltimore, D. 1983, "Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus", *Cell*, vol. 33, no. 1, pp. 153-159.

Marks, M. S., Roche, P. A., van Donselaar, E., Woodruff, L., & Peters, P. J. A Lysosomal Targeting Signal in the Cytoplasmic Tail of the Beta-Chain Directs HLA-DM to MHC Class II Compartments. J Biol.Chem. 2-10-1995.

Marks, M. S., Woodruff, L., Ohno, H., & Bonifacino, J. S. 1996, "Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components", *J Cell Biol.*, vol. 135, no. 2, pp. 341-354.

Mathis, D. & Benoist, C. 2007, "A decade of AIRE", *Nat Rev Immunol*, vol. 7, no. 8, pp. 645-650.

McCarty, D. M., Fu, H., Monahan, P. E., Toulson, C. E., Naik, P., & Samulski, R. J. 2003, "Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo", *Gene Ther*, vol. 10, no. 26, pp. 2112-2118.

McGeachy, M. J., Bak-Jensen, K. S., Chen, Y., Tato, C. M., Blumenschein, W., McClanahan, T., & Cua, D. J. 2007, "TGF-beta and IL-6 drive the production of

IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology", *Nat Immunol*, vol. 8, no. 12, pp. 1390-1397.

McKown, K. M., Carbone, L. D., Kaplan, S. B., Aelion, J. A., Lohr, K. M., Cremer, M. A., Bustillo, J., Gonzalez, M., Kaeley, G., Steere, E. L., Somes, G. W., Myers, L. K., Seyer, J. M., Kang, A. H., & Postlethwaite, A. E. 1999, "Lack of efficacy of oral bovine type II collagen added to existing therapy in rheumatoid arthritis", *Arthritis Rheum*, vol. 42, no. 6, pp. 1204-1208.

Mease, P. J., Hobbs, K., Chalmers, A., El-Gabalawy, H., Bookman, A., Keystone, E., Furst, D. E., Anklesaria, P., & Heald, A. E. 2009, "Local delivery of a recombinant adenoassociated vector containing a tumour necrosis factor alpha antagonist gene in inflammatory arthritis: a phase 1 dose-escalation safety and tolerability study", *Annals of the Rheumatic Diseases*, vol. 68, no. 8, pp. 1247-1254.

Medzhitov, R. 2001, "Toll-like receptors and innate immunity", *Nat.Rev.Immunol.*, vol. 1, no. 2, pp. 135-145.

Meffre, E. & Wardemann, H. 2008, "B-cell tolerance checkpoints in health and autoimmunity", *Curr.Opin.Immunol*, vol. 20, no. 6, pp. 632-638.

Michaelsson, E., Malmstrom, V., Reis, S., Engstrom, A., Burkhardt, H., & Holmdahl, R. 1994, "T cell recognition of carbohydrates on type II collagen", *J Exp.Med.*, vol. 180, no. 2, pp. 745-749.

Milici, A. J., Kudlacz, E. M., Audoly, L., Zwillich, S., & Changelian, P. 2008, "Cartilage preservation by inhibition of Janus kinase 3 in two rodent models of rheumatoid arthritis", *Arthritis Res.Ther.*, vol. 10, no. 1, p. R14.

Miller, A. D. & Buttimore, C. 1986, "Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production", *Mol.Cell Biol.*, vol. 6, no. 8, pp. 2895-2902.

Miller, D. G., Adam, M. A., & Miller, A. D. 1990, "Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection", *Mol.Cell Biol.*, vol. 10, no. 8, pp. 4239-4242.

Mitra, S. W., Goff, S., Gilboa, E., & Baltimore, D. 1979, "Synthesis of a 600-nucleotide-long plus-strand DNA by virions of Moloney murine leukemia virus", *Proc.Natl Acad.Sci.U.S.A*, vol. 76, no. 9, pp. 4355-4359.

Molling, K., Bolognesi, D. P., Bauer, H., Busen, W., Plassmann, H. W., & Hausen, P. 1971, "Association of viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA-DNA hybrids", *Nat New Biol.*, vol. 234, no. 51, pp. 240-243.

Mongkoldhumrongkul, N., Flanagan, J. M., & Jayasinghe, S. N. 2009, "Direct jetting approaches for handling stem cells", *Biomed.Mater.*, vol. 4, no. 1, p. 15018.

Montini, E., Cesana, D., Schmidt, M., Sanvito, F., Ponzoni, M., Bartholomae, C., Sergi, L. S., Benedicenti, F., Ambrosi, A., Di Serio, C., Doglioni, C., von Kalle, C., & Naldini, L. 2006, "Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration", *Nat Biotech*, vol. 24, no. 6, pp. 687-696.

Mosmann, T. R. & Coffman, R. L. 1989, "TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties", *Annu.Rev Immunol*, vol. 7, pp. 145-173.

Mowat, A. M., Parker, L. A., Beacock-Sharp, H., Millington, O. R., & Chirdo, F. 2004, "Oral tolerance: overview and historical perspectives", *Ann N Y.Acad.Sci.*, vol. 1029, pp. 1-8.

Mueller, D. L., Jenkins, M. K., & Schwartz, R. H. 1989, "Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy", *Annu.Rev Immunol*, vol. 7, pp. 445-480.

Mumm, S. R. & Grandgenett, D. P. 1991, "Defining nucleic acid-binding properties of avian retrovirus integrase by deletion analysis", *J Virol.*, vol. 65, no. 3, pp. 1160-1167.

Murphy, C. A., Langrish, C. L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R. A., Sedgwick, J. D., & Cua, D. J. 2003, "Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation", *J Exp.Med.*, vol. 198, no. 12, pp. 1951-1957.

Myers, L. K., Seyer, J. M., Stuart, J. M., Terato, K., David, C. S., & Kang, A. H. 1993, "T cell epitopes of type II collagen that regulate murine collagen-induced arthritis", *J.Immunol.*, vol. 151, no. 1, pp. 500-505.

Myers, L. K., Terato, K., Seyer, J. M., Stuart, J. M., & Kang, A. H. 1992, "Characterization of a tolerogenic T cell epitope of type II collagen and its relevance to collagen-induced arthritis", *J.Immunol.*, vol. 149, no. 4, pp. 1439-1443.

Nagamine, K., Peterson, P., Scott, H. S., Kudoh, J., Minoshima, S., Heino, M., Krohn, K. J. E., Lalioti, M. D., Mullis, P. E., Antonarakis, S. E., Kawasaki, K., Asakawa, S., Ito, F., & Shimizu, N. 1997, "Positional cloning of the APECED gene", *Nat Genet*, vol. 17, no. 4, pp. 393-398.

Nakae, S., Nambu, A., Sudo, K., & Iwakura, Y. 2003, "Suppression of Immune Induction of Collagen-Induced Arthritis in IL-17-Deficient Mice", *The Journal of Immunology*, vol. 171, no. 11, pp. 6173-6177.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M., & Trono, D. 1996, "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector", *Science*, vol. 272, no. 5259, pp. 263-267.

Nandakumar, K. S. & Holmdahl, R. 2006, "Antibody-induced arthritis: disease mechanisms and genes involved at the effector phase of arthritis", *Arthritis Res.Ther.*, vol. 8, no. 6, p. 223.

Nemazee, D. A. & Burki, K. 1989, "Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes", *Nature*, vol. 337, no. 6207, pp. 562-566.

Nermut, M. V. & Fassati, A. 2003, "Structural analyses of purified human immunodeficiency virus type 1 intracellular reverse transcription complexes", *J Virol.*, vol. 77, no. 15, pp. 8196-8206.

Neville, M., Stutz, F., Lee, L., Davis, L. I., & Rosbash, M. 1997, "The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export", *Curr.Biol.*, vol. 7, no. 10, pp. 767-775.

Nisole, S. & Saib, A. 2004, "Early steps of retrovirus replicative cycle", *Retrovirology.*, vol. 1, p. 9.

Norman, P. S., Nicodemus, C. F., Creticos, P. S., Wood, R. A., Eggleston, P. A., Lichtenstein, L. M., Kagey-Sobotka, A., & Proud, D. 1997, "Clinical and immunologic effects of component peptides in Allervax Cat", *Int.Arch.Allergy Immunol*, vol. 113, no. 1-3, pp. 224-226.

Nossal, G. J. 1994, "Negative selection of lymphocytes", *Cell*, vol. 76, no. 2, pp. 229-239.

Novy, P., Quigley, M., Huang, X., & Yang, Y. 2007, "CD4 T cells are required for CD8 T cell survival during both primary and memory recall responses", *J.Immunol.*, vol. 179, no. 12, pp. 8243-8251.

Nunes, F. A., Furth, E. E., Wilson, J. M., & Raper, S. E. 1999, "Gene transfer into the liver of nonhuman primates with E1-deleted recombinant adenoviral vectors: safety of readministration", *Hum.Gene Ther*, vol. 10, no. 15, pp. 2515-2526.

O'Garra, A. & Vieira, P. 2004, "Regulatory T cells and mechanisms of immune system control", *Nat Med.*, vol. 10, no. 8, pp. 801-805.

Ohnishi, Y., Tsutsumi, A., Sakamaki, T., & Sumida, T. 2003, "T cell epitopes of type II collagen in HLA-DRB1*0101 or DRB1*0405-positive Japanese patients with rheumatoid arthritis", *Int.J Mol.Med*, vol. 11, no. 3, pp. 331-335.

Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F., & Kastelein, R. A. 2000, "Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12", *Immunity*, vol. 13, no. 5, pp. 715-725.

Ott, M. G., Schmidt, M., Schwarzwaelder, K., Stein, S., Siler, U., Koehl, U., Glimm, H., Kuhlcke, K., Schilz, A., Kunkel, H., Naundorf, S., Brinkmann, A., Deichmann, A., Fischer, M., Ball, C., Pilz, I., Dunbar, C., Du, Y., Jenkins, N. A., Copeland, N. G., Luthi, U., Hassan, M., Thrasher, A. J., Hoelzer, D., Von, K. C., Seger, R., & Grez, M. 2006, "Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1", *Nat Med*, vol. 12, no. 4, pp. 401-409.

Pandiyan, P., Zheng, L., Ishihara, S., Reed, J., & Lenardo, M. J. 2007, "CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells", *Nat Immunol*, vol. 8, no. 12, pp. 1353-1362.

Pape, K. A., Merica, R., Mondino, A., Khoruts, A., & Jenkins, M. K. 1998, "Direct evidence that functionally impaired CD4+ T cells persist in vivo following induction of peripheral tolerance", *The Journal of Immunology*, vol. 160, no. 10, pp. 4719-4729.

Parada, C. A. & Roeder, R. G. 1996, "Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carboxy-terminal domain", *Nature*, vol. 384, no. 6607, pp. 375-378.

Parham, C., Chirica, M., Timans, J., Vaisberg, E., Travis, M., Cheung, J., Pflanz, S., Zhang, R., Singh, K. P., Vega, F., To, W., Wagner, J., O'Farrell, A. M., McClanahan, T., Zurawski, S., Hannum, C., Gorman, D., Rennick, D. M., Kastelein, R. A., de Waal, M. R., & Moore, K. W. 2002, "A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R", *The Journal of Immunology*, vol. 168, no. 11, pp. 5699-5708.

Park, J. Y. & Pillinger, M. H. 2007, "Interleukin-6 in the pathogenesis of rheumatoid arthritis", *Bull.NYU.Hosp.Jt.Dis.*, vol. 65 Suppl 1, pp. S4-10.

Parker, D. C. 1993, "T cell-dependent B cell activation", *Annu.Rev.Immunol.*, vol. 11, pp. 331-360.

Peng, Z. 2005, "Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers", *Hum.Gene Ther*, vol. 16, no. 9, pp. 1016-1027.

Peters, G., Harada, F., Dahlberg, J. E., Panet, A., Haseltine, W. A., & Baltimore, D. 1977, "Low-molecular-weight RNAs of Moloney murine leukemia virus: identification of the primer for RNA-directed DNA synthesis", *J Virol.*, vol. 21, no. 3, pp. 1031-1041.

Picca, C. C. & Caton, A. J. 2005, "The role of self-peptides in the development of CD4+ CD25+ regulatory T cells", *Curr.Opin.Immunol.*, vol. 17, no. 2, pp. 131-136.

Pike-Overzet, K., de, R. D., Weerkamp, F., Baert, M. R., Verstegen, M. M., Brugman, M. H., Howe, S. J., Reinders, M. J., Thrasher, A. J., Wagemaker, G., van Dongen, J. J., & Staal, F. J. 2007, "Ectopic retroviral expression of LMO2, but not IL2Rgamma, blocks human T-cell development from CD34+ cells: implications for leukemogenesis in gene therapy", *Leukemia*, vol. 21, no. 4, pp. 754-763.

Potter, P. K., Copier, J., Sacks, S. H., Calafat, J., Janssen, H., Neefjes, J. J., & Kelly, A. P. 1999, "Accurate intracellular localization of HLA-DM requires correct spacing of a cytoplasmic YTPL targeting motif relative to the transmembrane domain", *Eur.J Immunol*, vol. 29, no. 12, pp. 3936-3944.

Powrie, F., Carlino, J., Leach, M. W., Mauze, S., & Coffman, R. L. 1996, "A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells", *J Exp.Med.*, vol. 183, no. 6, pp. 2669-2674.

Quill, H. & Schwartz, R. H. 1987, "Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness", *The Journal of Immunology*, vol. 138, no. 11, pp. 3704-3712.

Ramsburg, E. A., Publicover, J. M., Coppock, D., & Rose, J. K. 2007, "Requirement for CD4 T cell help in maintenance of memory CD8 T cell responses is epitope dependent", *J.Immunol.*, vol. 178, no. 10, pp. 6350-6358.

Ranges, G. E., Sriram, S., & Cooper, S. M. 1985, "Prevention of type II collagen-induced arthritis by in vivo treatment with anti-L3T4", *J Exp.Med.*, vol. 162, no. 3, pp. 1105-1110.

Raper, S. E., Haskal, Z. J., Ye, X., Pugh, C., Furth, E. E., Gao, G. P., & Wilson, J. M. 1998, "Selective gene transfer into the liver of non-human primates with E1-deleted,

E2A-defective, or E1-E4 deleted recombinant adenoviruses", *Hum.Gene Ther*, vol. 9, no. 5, pp. 671-679.

Raper, S. E., Yudkoff, M., Chirmule, N., Gao, G. P., Nunes, F., Haskal, Z. J., Furth, E. E., Propert, K. J., Robinson, M. B., Magosin, S., Simoes, H., Speicher, L., Hughes, J., Tazelaar, J., Wivel, N. A., Wilson, J. M., & Batshaw, M. L. 2002, "A pilot study of in vivo liver-directed gene transfer with an adenoviral vector in partial ornithine transcarbamylase deficiency", *Hum.Gene Ther*, vol. 13, no. 1, pp. 163-175.

Reiser, K., McCormick, R. J., & Rucker, R. B. 1992, "Enzymatic and nonenzymatic cross-linking of collagen and elastin", *FASEB J*, vol. 6, no. 7, pp. 2439-2449.

Retter, M. W. & Nemazee, D. 1998, "Receptor editing occurs frequently during normal B cell development", *J Exp.Med.*, vol. 188, no. 7, pp. 1231-1238.

Rohn, T. A., Boes, M., Wolters, D., Spindeldreher, S., Muller, B., Langen, H., Ploegh, H., Vogt, A. B., & Kropshofer, H. 2004, "Upregulation of the CLIP self peptide on mature dendritic cells antagonizes T helper type 1 polarization", *Nat.Immunol.*, vol. 5, no. 9, pp. 909-918.

Rosenberg, S. A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R. A., Moen, R., Karson, E. M., Lotze, M. T., Yang, J. C., Topalian, S. L., & . 1990, "Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction", *N.Engl.J Med.*, vol. 323, no. 9, pp. 570-578.

Rosloniec, E. F., Whittington, K. B., Brand, D. D., Myers, L. K., & Stuart, J. M. 1996, "Identification of MHC class II and TCR binding residues in the type II collagen immunodominant determinant mediating collagen-induced arthritis", *Cell Immunol*, vol. 172, no. 1, pp. 21-28.

Ruckrich, T., Brandenburg, J., Cansier, A., Muller, M., Stevanovic, S., Schilling, K., Wiederanders, B., Beck, A., Melms, A., Reich, M., Driessen, C., & Kalbacher, H. 2006, "Specificity of human cathepsin S determined by processing of peptide substrates and MHC class II-associated invariant chain", *Biol.Chem.*, vol. 387, no. 10-11, pp. 1503-1511.

Ruiz, P. J., Garren, H., Ruiz, I. U., Hirschberg, D. L., Nguyen, L. V., Karpuj, M. V., Cooper, M. T., Mitchell, D. J., Fathman, C. G., & Steinman, L. 1999, "Suppressive immunization with DNA encoding a self-peptide prevents autoimmune disease: modulation of T cell costimulation", *The Journal of Immunology*, vol. 162, no. 6, pp. 3336-3341.

Ruotsalainen, H., Sipila, L., Vapola, M., Sormunen, R., Salo, A. M., Uitto, L., Mercer, D. K., Robins, S. P., Risteli, M., Aszodi, A., Fassler, R., & Myllyla, R.

2006a, "Glycosylation catalyzed by lysyl hydroxylase 3 is essential for basement membranes", *J.Cell Sci.*, vol. 119, no. Pt 4, pp. 625-635.

Ruotsalainen, H., Sipila, L., Vapola, M., Sormunen, R., Salo, A. M., Uitto, L., Mercer, D. K., Robins, S. P., Risteli, M., Aszodi, A., Fassler, R., & Myllyla, R. 2006b, "Glycosylation catalyzed by lysyl hydroxylase 3 is essential for basement membranes", *J.Cell Sci.*, vol. 119, no. Pt 4, pp. 625-635.

Ruotsalainen, H., Sipila, L., Vapola, M., Sormunen, R., Salo, A. M., Uitto, L., Mercer, D. K., Robins, S. P., Risteli, M., Aszodi, A., Fassler, R., & Myllyla, R. 2006c, "Glycosylation catalyzed by lysyl hydroxylase 3 is essential for basement membranes", *J.Cell Sci.*, vol. 119, no. Pt 4, pp. 625-635.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., & Toda, M. 1995, "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases", *The Journal of Immunology*, vol. 155, no. 3, pp. 1151-1164.

Sallusto, F., Kremmer, E., Palermo, B., Hoy, A., Ponath, P., Qin, S., Forster, R., Lipp, M., & Lanzavecchia, A. 1999, "Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells", *Eur.J.Immunol.*, vol. 29, no. 6, pp. 2037-2045.

Salo, A. M., Sipila, L., Sormunen, R., Ruotsalainen, H., Vainio, S., & Myllyla, R. 2006, "The lysyl hydroxylase isoforms are widely expressed during mouse embryogenesis, but obtain tissue- and cell-specific patterns in the adult", *Matrix Biol.*, vol. 25, no. 8, pp. 475-483.

Sant, S. M., Suarez, T. M., Moalli, M. R., Wu, B. Y., Blaivas, M., Laing, T. J., & Roessler, B. J. 1998, "Molecular lysis of synovial lining cells by in vivo herpes simplex virus-thymidine kinase gene transfer", *Hum.Gene Ther*, vol. 9, no. 18, pp. 2735-2743.

Santambrogio, L. & Strominger, J. L. 2006, "The Ins and Outs of MHC Class II Proteins in Dendritic Cells", *Immunity*, vol. 25, no. 6, pp. 857-859.

Schambach, A., Galla, M., Maetzig, T., Loew, R., & Baum, C. 2007, "Improving transcriptional termination of self-inactivating gamma-retroviral and lentiviral vectors", *Mol.Ther*, vol. 15, no. 6, pp. 1167-1173.

Schegg, B., Hulsmeier, A. J., Rutschmann, C., Maag, C., & Hennet, T. 2009, "Core glycosylation of collagen is initiated by two beta(1-O)galactosyltransferases", *Mol.Cell Biol.*, vol. 29, no. 4, pp. 943-952.

Schulte, S., Unger, C., Mo, J. A., Wendler, O., Bauer, E., Frischholz, S., von der, M. K., Kalden, J. R., Holmdahl, R., & Burkhardt, H. 1998, "Arthritis-related B cell epitopes in collagen II are conformation-dependent and sterically privileged in accessible sites of cartilage collagen fibrils", *J Biol.Chem.*, vol. 273, no. 3, pp. 1551-1561.

Schwartz, R. H. 2003, "T cell anergy", *Annu.Rev Immunol*, vol. 21, pp. 305-334.

Schwartz, S., Felber, B. K., Fenyo, E. M., & Pavlakis, G. N. 1990, "Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs", *The Journal of Virology*, vol. 64, no. 11, pp. 5448-5456.

Seeger, D. R., Cosulich, D. B., Smith, J. M., & Hultquist, M. E. 1949, "Analogs of Pteroylglutamic Acid. III. 4-Amino Derivatives", *Journal of the American Chemical Society*, vol. 71, no. 5, pp. 1753-1758.

Shahrara, S., Huang, Q., Mandelin, A. M., & Pope, R. M. 2008, "TH-17 cells in rheumatoid arthritis", *Arthritis Res.Ther.*, vol. 10, no. 4, p. R93.

Shan, S. o. & Walter, P. 2005, "Co-translational protein targeting by the signal recognition particle", *FEBS Letters*, vol. 579, no. 4, pp. 921-926.

Shibagaki, Y. & Chow, S. A. 1997, "Central core domain of retroviral integrase is responsible for target site selection", *J Biol.Chem.*, vol. 272, no. 13, pp. 8361-8369.

Shinnick, T. M., Lerner, R. A., & Sutcliffe, J. G. 1981, "Nucleotide sequence of Moloney murine leukaemia virus", *Nature*, vol. 293, no. 5833, pp. 543-548.

Sirven, A., Pflumio, F., Zennou, V., Titeux, M., Vainchenker, W., Coulombel, L., Dubart-Kupperschmitt, A., & Charneau, P. 2000, "The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells", *Blood*, vol. 96, no. 13, pp. 4103-4110.

Slavin, S., Strober, S., Fuks, Z., & Kaplan, H. S. 1977, "Induction of specific tissue transplantation tolerance using fractionated total lymphoid irradiation in adult mice: long-term survival of allogeneic bone marrow and skin grafts", *J Exp.Med.*, vol. 146, no. 1, pp. 34-48.

Sloan-Lancaster, J. & Allen, P. M. 1996, "Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology", *Annu.Rev Immunol*, vol. 14, pp. 1-27.

Smith, A. J., Bainbridge, J. W., & Ali, R. R. 2009, "Prospects for retinal gene replacement therapy", *Trends in Genetics*, vol. 25, no. 4, pp. 156-165.

Smith, J. K., Cywinski, A., & Taylor, J. M. 1984, "Specificity of initiation of plus-strand DNA by Rous sarcoma virus", *J Virol.*, vol. 52, no. 2, pp. 314-319.

Sojka, D. K., Huang, Y. H., & Fowell, D. J. 2008, "Mechanisms of regulatory T-cell suppression - a diverse arsenal for a moving target", *Immunology*, vol. 124, no. 1, pp. 13-22.

Song, L., Wang, J., Wang, R., Yu, M., Sun, Y., Han, G., Li, Y., Qian, J., Scott, D. W., Kang, Y., Soukhareva, N., & Shen, B. 2004, "Retroviral delivery of GAD-IgG fusion construct induces tolerance and modulates diabetes: a role for CD4+ regulatory T cells and TGF-beta?", *Gene Ther*, vol. 11, no. 20, pp. 1487-1496.

Song, X., Liang, F., Liu, N., Luo, Y., Xue, H., Yuan, F., Tan, L., Sun, Y., Xi, C., & Xi, Y. 2009, "Construction and characterization of a novel DNA vaccine that is potent antigen-specific tolerizing therapy for experimental arthritis by increasing CD4+CD25+Treg cells and inducing Th1 to Th2 shift in both cells and cytokines", *Vaccine*, vol. 27, no. 5, pp. 690-700.

Spiro, R. G. 1967, "The structure of the disaccharide unit of the renal glomerular basement membrane", *J Biol.Chem.*, vol. 242, no. 20, pp. 4813-4823.

Staffa, A. & Cochrane, A. 1994, "The tat/rev intron of human immunodeficiency virus type 1 is inefficiently spliced because of suboptimal signals in the 3' splice site", *J Virol.*, vol. 68, no. 5, pp. 3071-3079.

Staines, N. A., Derry, C. J., Marinova-Mutafchieva, L., Ali, N., Davies, D. H., & Murphy, J. J. 2004, "Constraints on the efficacy of mucosal tolerance in treatment of human and animal arthritic diseases", *Ann N Y.Acad.Sci.*, vol. 1029, pp. 250-259.

Stein, B. S. & Engleman, E. G. 1990, "Intracellular processing of the gp160 HIV-1 envelope precursor. Endoproteolytic cleavage occurs in a cis or medial compartment of the Golgi complex", *J Biol.Chem.*, vol. 265, no. 5, pp. 2640-2649.

Steinman, R. M. & Nussenzweig, M. C. 2002, "Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance", *Proc.Natl.Acad.Sci.U.S.A*, vol. 99, no. 1, pp. 351-358.

Stout, R. D. & Bottomly, K. 1989, "Antigen-specific activation of effector macrophages by IFN-gamma producing (TH1) T cell clones. Failure of IL-4-producing (TH2) T cell clones to activate effector function in macrophages", *J.Immunol.*, vol. 142, no. 3, pp. 760-765.

Strauss, L., Bergmann, C., Szczepanski, M., Gooding, W., Johnson, J. T., & Whiteside, T. L. 2007, "A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment", *Clin.Cancer Res.*, vol. 13, no. 15 Pt 1, pp. 4345-4354.

Strauss, G., Osen, W., & Debatin, K. M. 2002, "Induction of apoptosis and modulation of activation and effector function in T cells by immunosuppressive drugs", *Clin.Exp.Immunol*, vol. 128, no. 2, pp. 255-266.

Sugamura, K., Asao, H., Kondo, M., Tanaka, N., Ishii, N., Ohbo, K., Nakamura, M., & Takeshita, T. 1996, "THE INTERLEUKIN-2 RECEPTOR +¦ CHAIN: Its Role in the Multiple Cytokine Receptor Complexes and T Cell Development in XSCID", *Annual Review of Immunology*, vol. 14, no. 1, p. 179.

Sukiennicki, T. L. & Fowell, D. J. 2006, "Distinct Molecular Program Imposed on CD4+ T Cell Targets by CD4+CD25+ Regulatory T Cells", *The Journal of Immunology*, vol. 177, no. 10, pp. 6952-6961.

Surh, C. D. & Sprent, J. 1994, "T-cell apoptosis detected in situ during positive and negative selection in the thymus", *Nature*, vol. 372, no. 6501, pp. 100-103.

Svensson, L., Jirholt, J., Holmdahl, R., & Jansson, L. 1998, "B cell-deficient mice do not develop type II collagen-induced arthritis (CIA)", *Clin.Exp.Immunol*, vol. 111, no. 3, pp. 521-526.

Swee, L. K., Bosco, N., Malissen, B., Ceredig, R., & Rolink, A. 2009, "Expansion of peripheral naturally occurring T regulatory cells by Fms-like tyrosine kinase 3 ligand treatment", *Blood*, vol. 113, no. 25, pp. 6277-6287.

Sykes, M., Sachs, D. H., Nienhuis, A. W., Pearson, D. A., Moulton, A. D., & Bodine, D. M. 1993, "Specific prolongation of skin graft survival following retroviral transduction of bone marrow with an allogeneic major histocompatibility complex gene", *Transplantation*, vol. 55, no. 1, pp. 197-202.

Tada, Y., Ho, A., Koh, D. R., & Mak, T. W. 1996, "Collagen-induced arthritis in CD4- or CD8-deficient mice: CD8+ T cells play a role in initiation and regulate recovery phase of collagen-induced arthritis", *The Journal of Immunology*, vol. 156, no. 11, pp. 4520-4526.

Tadokoro, C. E., Shakhar, G., Shen, S., Ding, Y., Lino, A. C., Maraver, A., Lafaille, J. J., & Dustin, M. L. 2006, "Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo", *J Exp.Med.*, vol. 203, no. 3, pp. 505-511.

Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J., & Sakaguchi, S. 1998, "Immunologic self-tolerance maintained by CD25+CD4+

naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state", *Int.Immunol*, vol. 10, no. 12, pp. 1969-1980.

Tanaka, M., Ueno, T., Nakahara, T., Sasaki, K., Ishimoto, A., & Sakai, H. 2003, "Downregulation of CD4 is required for maintenance of viral infectivity of HIV-1", *Virology*, vol. 311, no. 2, pp. 316-325.

Terato, K., Hasty, K. A., Cremer, M. A., Stuart, J. M., Townes, A. S., & Kang, A. H. 1985, "Collagen-induced arthritis in mice. Localization of an arthritogenic determinant to a fragment of the type II collagen molecule", *The Journal of Experimental Medicine*, vol. 162, no. 2, pp. 637-646.

Thompson, R. N., Watts, C., Edelman, J., Esdaile, J., & Russell, A. S. 1984, "A controlled two-centre trial of parenteral methotrexate therapy for refractory rheumatoid arthritis", *J Rheumatol.*, vol. 11, no. 6, pp. 760-763.

Thomson, A. W. & Robbins, P. D. 2008, "Tolerogenic dendritic cells for autoimmune disease and transplantation", *Annals of the Rheumatic Diseases*, vol. 67 Suppl 3, p. iii90-iii96.

Thornton, A. M. & Shevach, E. M. 1998, "CD4+CD25+ Immunoregulatory T Cells Suppress Polyclonal T Cell Activation In Vitro by Inhibiting Interleukin 2áProduction", *The Journal of Experimental Medicine*, vol. 188, no. 2, pp. 287-296.

Thorstenson, K. M. & Khoruts, A. 2001, "Generation of anergic and potentially immunoregulatory CD25+CD4 T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen", *The Journal of Immunology*, vol. 167, no. 1, pp. 188-195.

Thrasher, A. J., Hacein-Bey-Abina, S., Gaspar, H. B., Blanche, S., Davies, E. G., Parsley, K., Gilmour, K., King, D., Howe, S., Sinclair, J., Hue, C., Carlier, F., Von, K. C., de Saint, B. G., le, D. F., Fischer, A., & Cavazzana-Calvo, M. 2005, "Failure of SCID-X1 gene therapy in older patients", *Blood*, vol. 105, no. 11, pp. 4255-4257.

Tiegs, S. L., Russell, D. M., & Nemazee, D. 1993, "Receptor editing in self-reactive bone marrow B cells", *The Journal of Experimental Medicine*, vol. 177, no. 4, pp. 1009-1020.

Tisch, R., Wang, B., & Serreze, D. V. 1999, "Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent", *The Journal of Immunology*, vol. 163, no. 3, pp. 1178-1187.

Trent, R. J. & Alexander, I. E. 2006, "Gene therapy in sport", *Br.J Sports Med.*, vol. 40, no. 1, pp. 4-5.

Trentham, D. E. 1982, "Collagen arthritis as a relevant model for rheumatoid arthritis", *Arthritis Rheum.*, vol. 25, no. 8, pp. 911-916.

Trentham, D. E., Dynesius-Trentham, R. A., Orav, E. J., Combitchi, D., Lorenzo, C., Sewell, K. L., Hafler, D. A., & Weiner, H. L. 1993, "Effects of oral administration of type II collagen on rheumatoid arthritis", *Science*, vol. 261, no. 5129, pp. 1727-1730.

Trentham, D. E., Townes, A. S., & Kang, A. H. 1977, "Autoimmunity to type II collagen an experimental model of arthritis", *J Exp.Med.*, vol. 146, no. 3, pp. 857-868.

Trono, D. 1992, "Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses", *J Virol.*, vol. 66, no. 8, pp. 4893-4900.

Valtavaara, M., Szpirer, C., Szpirer, J., & Myllyla, R. 1998, "Primary structure, tissue distribution, and chromosomal localization of a novel isoform of lysyl hydroxylase (lysyl hydroxylase 3)", *J Biol.Chem.*, vol. 273, no. 21, pp. 12881-12886.

van Vliet, S. J., den, D. J., Gringhuis, S. I., Geijtenbeek, T. B., & van, K. Y. 2007, "Innate signaling and regulation of Dendritic cell immunity", *Curr.Opin.Immunol*, vol. 19, no. 4, pp. 435-440.

Van, M. B. & Debyser, Z. 2005, "HIV-1 integration: an interplay between HIV-1 integrase, cellular and viral proteins", *AIDS Rev*, vol. 7, no. 1, pp. 26-43.

Vargas, J., Jr., Gusella, G. L., Najfeld, V., Klotman, M. E., & Cara, A. 2004, "Novel integrase-defective lentiviral episomal vectors for gene transfer", *Hum.Gene Ther*, vol. 15, no. 4, pp. 361-372.

Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., & Stockinger, B. 2006, "TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells", *Immunity*, vol. 24, no. 2, pp. 179-189.

Vieira, P. L., Christensen, J. R., Minaee, S., O'Neill, E. J., Barrat, F. J., Boonstra, A., Barthlott, T., Stockinger, B., Wraith, D. C., & O'Garra, A. 2004, "IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells", *The Journal of Immunology*, vol. 172, no. 10, pp. 5986-5993.

Vogt, A. B., Arndt, S. O., Hammerling, G. J., & Kropshofer, H. 1999, "Quality control of MHC class II associated peptides by HLA-DM/H2-M", *Semin.Immunol*, vol. 11, no. 6, pp. 391-403.

von Herrath, M. G. & Harrison, L. C. 2003, "Antigen-induced regulatory T cells in autoimmunity", *Nat Rev Immunol*, vol. 3, no. 3, pp. 223-232.

Waehler, R., Russell, S. J., & Curiel, D. T. 2007, "Engineering targeted viral vectors for gene therapy", *Nat Rev Genet*, vol. 8, no. 8, pp. 573-587.

Wang, C., Kovanen, V., Raudasoja, P., Eskelinen, S., Pospiech, H., & Myllyla, R. 2009, "The glycosyltransferase activities of lysyl hydroxylase 3 (LH3) in the extracellular space are important for cell growth and viability", *J.Cell Mol.Med.*, vol. 13, no. 3, pp. 508-521.

Wang, C., Luosujarvi, H., Heikkinen, J., Risteli, M., Uitto, L., & Myllyla, R. 2002, "The third activity for lysyl hydroxylase 3: galactosylation of hydroxylysyl residues in collagens in vitro", *Matrix Biol.*, vol. 21, no. 7, pp. 559-566.

Wang, C., Valtavaara, M., & Myllyla, R. 2000, "Lack of collagen type specificity for lysyl hydroxylase isoforms", *DNA Cell Biol.*, vol. 19, no. 2, pp. 71-77.

Ward, E. M., Chan, E., Gustafsson, K., & Jayasinghe, S. N. Combining bioelectrospray with gene therapy: a novel biotechnique for the delivery of genetic material *via* living cells. Analyst . 27-2-2010.

Wardemann, H., Yurasov, S., Schaefer, A., Young, J. W., Meffre, E., & Nussenzweig, M. C. 2003, "Predominant autoantibody production by early human B cell precursors", *Science*, vol. 301, no. 5638, pp. 1374-1377.
Warren, K. G., Catz, I., & Wucherpfennig, K. W. 1997, "Tolerance induction to myelin basic protein by intravenous synthetic peptides containing epitope P85 VVHFFKNIVTP96 in chronic progressive multiple sclerosis", *J Neurol.Sci.*, vol. 152, no. 1, pp. 31-38.

Watson, W. C. & Townes, A. S. 1985, "Genetic susceptibility to murine collagen II autoimmune arthritis. Proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-MHC loci", *The Journal of Experimental Medicine*, vol. 162, no. 6, pp. 1878-1891.

Wehling P., Reinecke J., Baltzer A. W., Granrath M., Schulitz K. P., Schultz C., Krauspe R., Whiteside T. W., Elder E., Ghivizzani S. C., Robbins P. D., Evans C. H. 2009, “Clinical responses to gene therapy in joints of two subjects with rheumatoid arthritis”, *Hum Gene Ther.* 2009 Feb;20(2):97-101

Weinblatt, M. E., Coblyn, J. S., Fox, D. A., Fraser, P. A., Holdsworth, D. E., Glass, D. N., & Trentham, D. E. 1985, "Efficacy of low-dose methotrexate in rheumatoid arthritis", *N.Engl.J Med.*, vol. 312, no. 13, pp. 818-822.

Weiner, H. L. 2001, "Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells", *Immunol Rev*, vol. 182, pp. 207-214.

Wessels, J. A. M., Huizinga, T. W. J., & Guchelaar, H. J. 2008, "Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis", *Rheumatology*, vol. 47, no. 3, pp. 249-255.

Westerberg, L. S., Klein, C., & Snapper, S. B. 2008, "Breakdown of T cell tolerance and autoimmunity in primary immunodeficiency--lessons learned from monogenic disorders in mice and men", *Curr.Opin.Immunol*, vol. 20, no. 6, pp. 646-654.

White, H. N., ., Thrasher, A. J., Veys, P. A., Kinnon, C., & Gaspar, H. B. 2000, "Intrinsic defects of B cell function in X-linked severe combined immunodeficiency", *Lancet*, vol. 364, no. 9452, pp. 2181-2187.

Wilkinson, B. M., Regnacq, M., & Stirling, C. J. 1997, "Protein translocation across the membrane of the endoplasmic reticulum", *J Membr.Biol.*, vol. 155, no. 3, pp. 189-197.

Williams, R. O. 1998, "Rodent models of arthritis: relevance for human disease", *Clin.Exp.Immunol*, vol. 114, no. 3, pp. 330-332.

Wilson, J. M. 2009, "Lessons learned from the gene therapy trial for ornithine transcarbamylase deficiency", *Mol.Genet Metab*, vol. 96, no. 4, pp. 151-157.

Wodrich, H. & Krausslich, H. G. 2001, "Nucleocytoplasmic RNA transport in retroviral replication", *Results Probl.Cell Differ.*, vol. 34, pp. 197-217.

Wolff, J. A., Dowty, M. E., Jiao, S., Repetto, G., Berg, R. K., Ludtke, J. J., Williams, P., & Slautterback, D. B. 1992, "Expression of naked plasmids by cultured myotubes and entry of plasmids into T tubules and caveolae of mammalian skeletal muscle", *J Cell Sci.*, vol. 103 (Pt 4), pp. 1249-1259.

Wommack, K. E. & Colwell, R. R. 2000, "Virioplankton: Viruses in Aquatic Ecosystems", *Microbiology and Molecular Biology Reviews*, vol. 64, no. 1, pp. 69-114.

Woods, N. B., Bottero, V., Schmidt, M., von Kalle, C., & Verma, I. M. 2006, "Gene therapy: Therapeutic gene causing lymphoma", *Nature*, vol. 440, no. 7088, p. 1123.

Woodward, C. L., Prakobwanakit, S., Mosessian, S., & Chow, S. A. 2009, "Integrase Interacts with Nucleoporin NUP153 To Mediate the Nuclear Import of Human Immunodeficiency Virus Type 1", *The Journal of Virology*, vol. 83, no. 13, pp. 6522-6533.

Wooley, P. H., Luthra, H. S., Griffiths, M. M., Stuart, J. M., Huse, A., & David, C. S. 1985, "Type II collagen-induced arthritis in mice. IV. Variations in immunogenetic regulation provide evidence for multiple arthritogenic epitopes on the collagen molecule", *The Journal of Immunology*, vol. 135, no. 4, pp. 2443-2451.

Wooley, P. H., Luthra, H. S., Singh, S. K., Huse, A. R., Stuart, J. M., & David, C. S. 1984, "Passive transfer of arthritis to mice by injection of human anti-type II collagen antibody", *Mayo Clin.Proc.*, vol. 59, no. 11, pp. 737-743.

Wooley, P. H., Luthra, H. S., Stuart, J. M., & David, C. S. 1981, "Type II collagen-induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates", *J Exp.Med.*, vol. 154, no. 3, pp. 688-700.

Wu, T. C., Guarnieri, F. G., Staveley-O'Carroll, K. F., Viscidi, R. P., Levitsky, H. I., Hedrick, L., Cho, K. R., August, J. T., & Pardoll, D. M. 1995, "Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens", *Proc.Natl.Acad.Sci.U.S.A*, vol. 92, no. 25, pp. 11671-11675.

Yamashita, M. & Emerman, M. 2009, "Cellular restriction targeting viral capsids perturbs HIV-1 infection of non-dividing cells", *J Virol.*

Yamazaki, S., Iyoda, T., Tarbell, K., Olson, K., Velinzon, K., Inaba, K., & Steinman, R. M. 2003, "Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells", *J Exp.Med.*, vol. 198, no. 2, pp. 235-247.

Yanez-Munoz, R. J., Balaggan, K. S., MacNeil, A., Howe, S. J., Schmidt, M., Smith, A. J., Buch, P., MacLaren, R. E., Anderson, P. N., Barker, S. E., Duran, Y., Bartholomae, C., Von, K. C., Heckenlively, J. R., Kinnon, C., Ali, R. R., & Thrasher, A. J. 2006, "Effective gene therapy with nonintegrating lentiviral vectors", *Nat Med*, vol. 12, no. 3, pp. 348-353.

Yoshinaka, Y., Katoh, I., Copeland, T. D., & Oroszlan, S. 1985, "Murine leukemia virus protease is encoded by the gag-pol gene and is synthesized through suppression of an amber termination codon", *Proc.Natl Acad.Sci.U.S.A*, vol. 82, no. 6, pp. 1618-1622.

Yoshino, S., Cleland, L. G., & Mayrhofer, G. 1991, "Treatment of collagen-induced arthritis in rats with a monoclonal antibody against the alpha/beta T cell antigen receptor", *Arthritis Rheum.*, vol. 34, no. 8, pp. 1039-1047.

Yu, S. F., von, R. T., Kantoff, P. W., Garber, C., Seiberg, M., Ruther, U., Anderson, W. F., Wagner, E. F., & Gilboa, E. 1986, "Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells", *Proc.Natl Acad.Sci.U.S.A*, vol. 83, no. 10, pp. 3194-3198.

Yu, Y., Iclozan, C., Yamazaki, T., Yang, X., Anasetti, C., Dong, C., & Yu, X. Z. 2009, "Abundant c-FLIP expression determines resistance of Th17 cells to activation-induced cell death", *Blood* p. blood-2009.

Zaiss, A. K., Son, S., & Chang, L. J. 2002, "RNA 3' readthrough of oncoretrovirus and lentivirus: implications for vector safety and efficacy", *J Virol.*, vol. 76, no. 14, pp. 7209-7219.

Zambidis, E. T. & Scott, D. W. 1996, "Epitope-specific tolerance induction with an engineered immunoglobulin", *Proc.Natl Acad.Sci.U.S.A*, vol. 93, no. 10, pp. 5019-5024.

Zan-Bar, I., Slavin, S., & Strober, S. 1978, "Induction and mechanism of tolerance to bovine serum albumin in mice given total lymphoid irradiation (TLI)", *The Journal of Immunology*, vol. 121, no. 4, pp. 1400-1404.

Zavada, J. 1972, "VSV pseudotype particles with the coat of avian myeloblastosis virus", *Nature New Biol.*, vol. 240, pp. 122-124.

Zhang, X. L., Peng, J., Sun, J. Z., Liu, J. J., Guo, C. S., Wang, Z. G., Yu, Y., Shi, Y., Qin, P., Li, S. G., Zhang, L. N., & Hou, M. 2009, "De novo induction of platelet-specific CD4+CD25+ regulatory T cells from CD4+CD25- cells in patients with idiopathic thrombocytopenic purpura", *Blood*, vol. 113, no. 11, pp. 2568-2577.

Zhang, Z. J., Davidson, L., Eisenbarth, G., & Weiner, H. L. 1991, "Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin", *Proc.Natl Acad.Sci.U.S.A*, vol. 88, no. 22, pp. 10252-10256.

Zheng, R., Jenkins, T. M., & Craigie, R. 1996, "Zinc folds the N-terminal domain of HIV-1 integrase, promotes multimerization, and enhances catalytic activity", *Proc.Natl Acad.Sci.U.S.A*, vol. 93, no. 24, pp. 13659-13664.

Zheng, S. G., Gray, J. D., Ohtsuka, K., Yamagiwa, S., & Horwitz, D. A. 2002, "Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors", *The Journal of Immunology*, vol. 169, no. 8, pp. 4183-4189.

Zheng, S. G., Wang, J. H., Gray, J. D., Soucier, H., & Horwitz, D. A. 2004, "Natural and induced CD4+CD25+ cells educate CD4+", *The Journal of Immunology*, vol. 172, no. 9, pp. 5213-5221.

Zheng, S. G., Wang, J. H., Stohl, W., Kim, K. S., Gray, J. D., & Horwitz, D. A. 2006, "TGF-beta Requires CTLA-4 Early after T Cell Activation to Induce FoxP3 and Generate Adaptive CD4+CD25+ Regulatory Cells", *The Journal of Immunology*, vol. 176, no. 6, pp. 3321-3329.

Zhou, X. Y., Yashiro-Ohtani, Y., Nakahira, M., Park, W. R., Abe, R., Hamaoka, T., Naramura, M., Gu, H., & Fujiwara, H. 2002, "Molecular mechanisms underlying differential contribution of CD28 versus non-CD28 costimulatory molecules to IL-2 promoter activation", *J.Immunol.*, vol. 168, no. 8, pp. 3847-3854.

Zhu, P., Li, X. Y., Wang, H. K., Jia, J. F., Zheng, Z. H., Ding, J., & Fan, C. M. 2007, "Oral administration of type-II collagen peptide 250-270 suppresses specific cellular and humoral immune response in collagen-induced arthritis", *Clin.Immunol*, vol. 122, no. 1, pp. 75-84.

Zufferey, R., Donello, J. E., Trono, D., & Hope, T. J. 1999, "Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors", *J Virol.*, vol. 73, no. 4, pp. 2886-2892.

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., & Trono, D. 1998, "Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery", *J Virol.*, vol. 72, no. 12, pp. 9873-9880.

Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L., & Trono, D. 1997, "Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo", *Nat Biotechnol.*, vol. 15, no. 9, pp. 871-875.

Sequences

DNA Sequence Translated - DNADynamo File pLssGCH12
Page 1 of 3

1 CTT TAA GAC CAA TGA CTT ACA AGG CAG CTG TAG ATC TTA GCC ACT TTT TAA AAG AAA AGG
61 GGG GAC TGG AAG GGC TAA TTC ACT CCC AAC GAA GAC AAG ATC TGC TTT TTG CTT GTA CTG
121 GGT CTC TCT GGT TAG ACC AGA TCT GAG CCT GGG AGC TCT CTG GCT AAC TAG GGA ACC CAC
181 TGC TTA AGC CTC AAT AAA GCT TGC CTT GAG TGC TTC AAG TAG TGT GTG CCC GTC TGT TGT
241 GTG ACT CTG GTA ACT AGA GAT CCC TCA GAC CCT TTT AGT CAG TGT GGA AAA TCT CTA GCA
301 GCA TCT AGA ATT AAT TCC GTG TAT TCT ATA GTG TCA CCT AAA TCG TAT GTG TAT GAT ACA
361 TAA GGT TAT GTA TTA ATT GTA GCC GCG TTC TAA CGA CAA TAT GTA CAA GCC TAA TTG TGT
421 AGC ATC TGG CTT ACT GAA GCA GAC CCT ATC ATC TCT CTC GTA AAC TGC CGT CAG AGT CGG
481 TTT GGT TGG ACG AAC CTT CTG AGT TTC TGG TAA CGC CGT CCC GCA CCC GGA AAT GGT CAG
541 CGA ACC AAT CAG CAG GGT CAT CGC TAG CCA GAT CCT CTA CGC CGG ACG CAT CGT GGC CGG
601 CAT CAC CGG CGC CAC AGG TGC GGT TGC TGG CGC CTA TAT CGC CGA CAT CAC CGA TGG GGA
661 AGA TCG GGC TCG CCA CTT CGG GCT CAT GAG CGC TTG TTT CGG CGT GGG TAT GGT GGC AGG
721 CCC CGT GGC CGG GGG ACT GTT GGG CGC CAT CTC CTT GCA TGC ACC ATT CCT TGC GGC GGC
781 GGT GCT CAA CGG CCT CAA CCT ACT ACT GGG CTG CTT CCT AAT GCA GGA GTC GCA TAA GGG
841 AGA GCG TCG AAT GGT GCA CTC TCA GTA CAA TCT GCT CTG ATG CCG CAT AGT TAA GCC AGC
901 CCC GAC ACC CGC CAA CAC CCG CTG ACG CGC CCT GAC GGG CTT GTC TGC TCC CGG CAT CCG
961 CTT ACA GAC AAG CTG TGA CCG TCT CCG GGA GCT GCA TGT GTC AGA GGT TTT CAC CGT CAT
1021 CAC CGA AAC GCG CGA GAC GAA AGG GCC TCG TGA TAC GCC TAT TTT TAT AGG TTA ATG TCA
1081 TGA TAA TAA TGG TTT CTT AGA CGT CAG GTG GCA CTT TTC GGG GAA ATG TGC GCG GAA CCC
1141 CTA TTT GTT TAT TTT TCT AAA TAC ATT CAA ATA TGT ATC CGC TCA TGA GAC AAT AAC CCT
1201 GAT AAA TGC TTC AAT AAT ATT GAA AAA GGA AGA GTA TGA GTA TTC AAC ATT TCC GTG TCG
1261 CCC TTA TTC CCT TTT TTG CGG CAT TTT GCC TTC CTG TTT TTG CTC ACC CAG AAA CGC TGG
1321 TGA AAG TAA AAG ATG CTG AAG ATC AGT TGG GTG CAC GAG TGG GTT ACA TCG AAC TGG ATC
1381 TCA ACA GCG GTA AGA TCC TTG AGA GTT TTC GCC CCG AAG AAC GTT TTC CAA TGA TGA GCA
1441 CTT TTA AAG TTC TGC TAT GTG GCG CGG TAT TAT CCC GTA TTG ACG CCG GGC AAG AGC AAC
1501 TCG GTC GCC GCA TAC ACT ATT CTC AGA ATG ACT TGG TTG AGT ACT CAC CAG TCA CAG AAA
1561 AGC ATC TTA CGG ATG GCA TGA CAG TAA GAG AAT TAT GCA GTG CTG CCA TAA CCA TGA GTG
1621 ATA ACA CTG CGG CCA ACT TAC TTC TGA CAA CGA TCG GAG GAC CGA AGG AGC TAA CCG CTT
1681 TTT TGC ACA ACA TGG GGG ATC ATG TAA CTC GCC TTG ATC GTT GGG AAC CGG AGC TGA ATG
1741 AAG CCA TAC CAA ACG ACG AGC GTG ACA CCA CGA TGC CTG TAG CAA TGG CAA CAA CGT TGC
1801 GCA AAC TAT TAA CTG GCG AAC TAC TTA CTC TAG CTT CCC GGC AAC AAT TAA TAG ACT GGA
1861 TGG AGG CGG ATA AAG TTG CAG GAC CAC TTC TGC GCT CGG CCC TTC CGG CTG GCT GGT TTA
1921 TTG CTG ATA AAT CTG GAG CCG GTG AGC GTG GGT CTC GCG GTA TCA TTG CAG CAC TGG GGC
1981 CAG ATG GTA AGC CCT CCC GTA TCG TAG TTA TCT ACA CGA CGG GGA GTC AGG CAA CTA TGG
2041 ATG AAC GAA ATA GAC AGA TCG CTG AGA TAG GTG CCT CAC TGA TTA AGC ATT GGT AAC TGT
2101 CAG ACC AAG TTT ACT CAT ATA TAC TTT AGA TTG ATT TAA AAC TTC ATT TTT AAT TTA AAA
2161 GGA TCT AGG TGA AGA TCC TTT TTG ATA ATC TCA TGA CCA AAA TCC CTT AAC GTG AGT TTT
2221 CGT TCC ACT GAG CGT CAG ACC CCG TAG AAA AGA TCA AAG GAT CTT CTT GAG ATC CTT TTT
2281 TTC TGC GCG TAA TCT GCT GCT TGC AAA CAA AAA AAC CAC CGC TAC CAG CGG TGG TTT GTT
2341 TGC CGG ATC AAG AGC TAC CAA CTC TTT TTC CGA AGG TAA CTG GCT TCA GCA GAG CGC AGA
2401 TAC CAA ATA CTG TCC TTC TAG TGT AGC CGT AGT TAG GCC ACC ACT TCA AGA ACT CTG TAG
2461 CAC CGC CTA CAT ACC TCG CTC TGC TAA TCC TGT TAC CAG TGG CTG CTG CCA GTG GCG ATA
2521 AGT CGT GTC TTA CCG GGT TGG ACT CAA GAC GAT AGT TAC CGG ATA AGG CGC AGC GGT CGG
2581 GCT GAA CGG GGG GTT CGT GCA CAC AGC CCA GCT TGG AGC GAA CGA CCT ACA CCG AAC TGA
2641 GAT ACC TAC AGC GTG AGC ATT GAG AAA GCG CCA CGC TTC CCG AAG GGA GAA AGG CGG ACA
2701 GGT ATC CGG TAA GCG GCA GGG TCG GAA CAG GAG AGC GCA CGA GGG AGC TTC CAG GGG GAA
2761 ACG CCT GGT ATC TTT ATA GTC CTG TCG GGT TTC GCC ACC TCT GAC TTG AGC GTC GAT TTT
2821 TGT GAT GCT CGT CAG GGG GGC GGA GCC TAT GGA AAA ACG CCA GCA ACG CGG CCT TTT TAC
2881 GGT TCC TGG CCT TTT GCT GGC CTT TTG CTC ACA TGT TCT TTC CTG CGT TAT CCC CTG ATT
2941 CTG TGG ATA ACC GTA TTA CCG CCT TTG AGT GAG CTG ATA CCG CTC GCC GCA GCC GAA CGA
3001 CCG AGC GCA GCG AGT CAG TGA GCG AGG AAG CGG AAG AGC GCC CAA TAC GCA AAC CGC CTC
3061 TCC CCG CGC GTT GGC CGA TTC ATT AAT GCA GCT GTG GAA TGT GTG TCA GTT AGG GTG TGG
3121 AAA GTC CCC AGG CTC CCC AGC AGG CAG AAG TAT GCA AAG CAT GCA TCT CAA TTA GTC AGC
3181 AAC CAG GTG TGG AAA GTC CCC AGG CTC CCC AGC AGG CAG AAG TAT GCA AAG CAT GCA TCT
3241 CAA TTA GTC AGC AAC CAT AGT CCC GCC CCT AAC TCC GCC CAT CCC GCC CCT AAC TCC GCC
3301 CAG TTC CGC CCA TTC TCC GCC CCA TGG CTG ACT AAT TTT TTT TAT TTA TGC AGA GGC CGA
3361 GGC CGC CTC GGC CTC TGA GCT ATT CCA GAA GTA GTG AGG AGG CTT TTT TGG AGG CCT AGG
3421 CTT TTG CAA AAA GCT TGG ACA CAA GAC AGG CTT GCG AGA TAT GTT TGA GAA TAC CAC TTT
3481 ATC CCG CGT CAG GGA GAG GCA GTG CGT AAA AAG ACG CGG ACT CAT GTG AAA TAC TGG TTT
3541 TTA GTG CGC CAG ATC TCT ATA ATC TCG CGC AAC CTA TTT TCC CCT CGA ACA CTT TTT AAG
3601 CCG TAG ATA AAC AGG CTG GGA CAC TTC ACA TGA GCG AAA AAT ACA TCG TCA CCT GGG ACA

3661 TGT TGC AGA TCC ATG CAC GTA AAC TCG CAA GCC GAC TGA TGC CTT CTG AAC AAT GGA AAG
3721 GCA TTA TTG CCG TAA GCC GTG GCG GTC TGT ACC GGG TGC GTT ACT GGC GCG TGA ACT GGG
3781 TAT TCG TCA TGT CGA TAC CGT TTG TAT TTC CAG CTA CGA TCA CGA CAA CCA GCG CGA GCT
3841 TAA AGT GCT GAA ACG CGC AGA AGG CGA TGG CGA AGG CTT CAT CGT TAT TGA TGA CCT GGT
3901 GGA TAC CGG TGG TAC TGC GGT TGC GAT TCG TGA AAT GTA TCC AAA AGC GCA CTT TGT CAC
3961 CAT CTT CGC AAA ACC GGC TGG TCG TCC GCT GGT TGA TGA CTA TGT TGT TGA TAT CCC GCA
4021 AGA TAC CTG GAT TGA ACA GCC GTG GGA TAT GGG CGT CGT ATT CGT CCC GCC AAT CTC CGG
4081 TCG CTA ATC TTT TCA ACG CCT GGC ACT GCC GGG CGT TGT TCT TTT TAA CTT CAG GCG GGT
4141 TAC AAT AGT TTC CAG TAA GTA TTC TGG AGG CTG CAT CCA TGA CAC AGG CAA ACC TGA GCG
4201 AAA CCC TGT TCA AAC CCC GCT TTA AAC ATC CTG AAA CCT CGA CGC TAG TCC GCC GCT TTA
4261 ATC ACG GCG CAC AAC CGC CTG TGC AGT CGG CCC TTG ATG GTA AAA CCA TCC CTC ACT GGT
4321 ATC GCA TGA TTA ACC GTC TGA TGT GGA TCT GGC GCG GCA TTG ACC CAC GCG AAA TCC TCG
4381 ACG TCC AGG CAC GTA TTG TGA TGA GCG ATG CCG AAC GTA CCG ACG ATG ATT TAT ACG ATA
4441 CGG TGA TTG GCT ACC GTG GCG GCA ACT GGA TTT ATG AGT GGG CCC CGG ATC TTT GTG AAG
4501 GAA CCT TAC TTC TGT GGT GTG ACA TAA TTG GAC AAA CTA CCT ACA GAG ATT TAA AGC TCT
4561 AAG GTA AAT ATA AAA TTT TTA AGT GTA TAA TGT GTT AAA CTA CTG ATT CTA ATT GTT TGT
4621 GTA TTT TAG ATT CCA ACC TAT GGA ACT GAT GAA TGG GAG CAG TGG TGG AAT GCC TTT AAT
4681 GAG GAA AAC CTG TTT TGC TCA GAA GAA ATG CCA TCT AGT GAT GAT GAG GCT ACT GCT GAC
4741 TCT CAA CAT TCT ACT CCT CCA AAA AAG AAG AGA AAG GTA GAA GAC CCC AAG GAC TTT CCT
4801 TCA GAA TTG CTA AGT TTT TTG AGT CAT GCT GTG TTT AGT AAT AGA ACT CTT GCT TGC TTT
4861 GCT ATT TAC ACC ACA AAG GAA AAA GCT GCA CTG CTA TAC AAG AAA ATT ATG GAA AAA TAT
4921 TCT GTA ACC TTT ATA AGT AGG CAT AAC AGT TAT AAT CAT AAC ATA CTG TTT TTT CTT ACT
4981 CCA CAC AGG CAT AGA GTG TCT GCT ATT AAT AAC TAT GCT CAA AAA TTG TGT ACC TTT AGC
5041 TTT TTA ATT TGT AAA GGG GTT AAT AAG GAA TAT TTG ATG TAT AGT GCC TTG ACT AGA GAT
5101 CAT AAT CAG CCA TAC CAC ATT TGT AGA GGT TTT ACT TGC TTT AAA AAA CCT CCC ACA CCT
5161 CCC CCT GAA CCT GAA ACA TAA AAT GAA TGC AAT TGT TGT TGT TAA CTT GTT TAT TGC AGC
5221 TTA TAA TGG TTA CAA ATA AAG CAA TAG CAT CAC AAA TTT CAC AAA TAA AGC ATT TTT TTC
5281 ACT GCA TTC TAG TTG TGG TTT GTC CAA ACT CAT CAA TGT ATC TTA TCA TGT CTG GAT CAA
5341 CTG GAT AAC TCA AGC TAA CCA AAA TCA TCC CAA ACT TCC CAC CCC ATA CCC TAT TAC CAC
5401 TGC CAA TTA CCT AGT GGT TTC ATT TAC TCT AAA CCT GTG ATT CCT CTG AAT TAT TTT CAT
5461 TTT AAA GAA ATT GTA TTT GTT AAA TAT GTA CTA CAA ACT TAG TAG TTG GAA GGG CTA ATT
5521 CAC TCC CAA AGA AGA CAA GAT ATC CTT GAT CTG TGG ATC TAC CAC ACA CAA GGC TAC TTC
5581 CCT GAT TAG CAG AAC TAC ACA CCA GGG CCA GGG GTC AGA TAT CCA CTG ACC TTT GGA TGG
5641 TGC TAC AAG CTA GTA CCA GTT GAG CCA GAT AAG GTA GAA GAG GCC AAT AAA GGA GAG AAC
5701 ACC AGC TTG TTA CAC CCT GTG AGC CTG CAT GGG ATG GAT GAC CCG GAG AGA GAA GTG TTA
5761 GAG TGG AGG TTT GAC AGC CGC CTA GCA TTT CAT CAC GTG GCC CGA GAG CTG CAT CCG GAG
5821 TAC TTC AAG AAC TGC TGA TAT CGA GCT TGC TAC AAG GGA CTT TCC GCT GGG GAC TTT CCA
5881 GGG AGG CGT GGC CTG GGC GGG ACT GGG GAG TGG CGA GCC CTC AGA TCC TGC ATA TAA GCA
5941 GCT GCT TTT TGC CTG TAC TGG GTC TCT CTG GTT AGA CCA GAT CTG AGC CTG GGA GCT CTC
6001 TGG CTA ACT AGG GAA CCC ACT GCT TAA GCC TCA ATA AAG CTT GCC TTG AGT GCT TCA AGT
6061 AGT GTG TGC CCG TCT GTT GTG TGA CTC TGG TAA CTA GAG ATC CCT CAG ACC CTT TTA GTC
6121 AGT GTG GAA AAT CTC TAG CAG TGG CGC CCG AAC AGG GAC TTG AAA GCG AAA GGG AAA CCA
6181 GAG GAG CTC TCT CGA CGC AGG ACT CGG CTT GCT GAA GCG CGC ACG GCA AGA GGC GAG GGG
6241 CGG CGA CTG GTG AGT ACG CCA AAA ATT TTG ACT AGC GGA GGC TAG AAG GAG AGA GAT GGG
6301 TGC GAG AGC GTC AGT ATT AAG CGG GGG AGA ATT AGA TCG CGA TGG GAA AAA ATT CGG TTA
6361 AGG CCA GGG GGA AAG AAA AAA TAT AAA TTA AAA CAT ATA GTA TGG GCA AGC AGG GAG CTA
6421 GAA CGA TTC GCA GTT AAT CCT GGC CTG TTA GAA ACA TCA GAA GGC TGT AGA CAA ATA CTG
6481 GGA CAG CTA CAA CCA TCC CTT CAG ACA GGA TCA GAA GAA CTT AGA TCA TTA TAT AAT ACA
6541 GTA GCA ACC CTC TAT TGT GTG CAT CAA AGG ATA GAG ATA AAA GAC ACC AAG GAA GCT TTA
6601 GAC AAG ATA GAG GAA GAG CAA AAC AAA AGT AAG ACC ACC GCA CAG CAA GCG GCC GCT GAT
6661 CTT CAG ACC TGG AGG AGG AGA TAT GAG GGA CAA TTG GAG AAG TGA ATT ATA TAA ATA TAA
6721 AGT AGT AAA AAT TGA ACC ATT AGG AGT AGC ACC CAC CAA GGC AAA GAG AAG AGT GGT GCA
6781 GAG AGA AAA AAG AGC AGT GGG AAT AGG AGC TTT GTT CCT TGG GTT CTT GGG AGC AGC AGG
6841 AAG CAC TAT GGG CGC AGC GTC AAT GAC GCT GAC GGT ACA GGC CAG ACA ATT ATT GTC TGG
6901 TAT AGT GCA GCA GCA GAA CAA TTT GCT GAG GGC TAT TGA GGC GCA ACA GCA TCT GTT GCA
6961 ACT CAC AGT CTG GGG CAT CAA GCA GCT CCA GGC AAG AAT CCT GGC TGT GGA AAG ATA CCT
7021 AAA GGA TCA ACA GCT CCT GGG GAT TTG GGG TTG CTC TGG AAA ACT CAT TTG CAC CAC TGC
7081 TGT GCC TTG GAA TGC TAG TTG GAG TAA TAA ATC TCT GGA ACA GAT TTG GAA TCA CAC GAC
7141 CTG GAT GGA GTG GGA CAG AGA AAT TAA CAA TTA CAC AAG CTT AAT ACA CTC CTT AAT TGA
7201 AGA ATC GCA AAA CCA GCA AGA AAA GAA TGA ACA AGA ATT ATT GGA ATT AGA TAA ATG GGC
7261 AAG TTT GTG GAA TTG GTT TAA CAT AAC AAA TTG GCT GTG GTA TAT AAA ATT ATT CAT AAT
7321 GAT AGT AGG AGG CTT GGT AGG TTT AAG AAT AGT TTT TGC TGT ACT TTC TAT AGT GAA TAG
7381 AGT TAG GCA GGG ATA TTC ACC ATT ATC GTT TCA GAC CCA CCT CCC AAC CCC GAG GGG ACC
7441 CGA CAG GCC CGA AGG AAT AGA AGA AGA AGG TGG AGA GAG AGA CAG AGA CAG ATC CAT TCG

```
7501 ATT AGT GAA CGG ATC TCG ACG GTC GCC AAA TGG CAG TAT TCA TCC ACA ATT TTA AAA GAA
7561 AAG GGG GGA TTG GGG GGT ACA GTG CAG GGG AAA GAA TAG TAG ACA TAA TAG CAA CAG ACA
7621 TAC AAA CTA AAG AAT TAC AAA AAC AAA TTA CAA AAA TTC AAA ATT TTC GGG TTT ATT ACA
7681 GGG ACA GCA GAG ATC CAG TTT GGA TCG ATA AGC TTG ATA TCG AAT TCC TGC AGC CCC GAT
7741 AAA ATA AAA GAT TTT ATT TAG TCT CCA GAA AAA GGG GGG AAT GAA AGA CCC CAC CTG TAG
7801 GTT TGG CAA GCT AGC TGC AGT AAC GCC ATT TTG CAA GGC ATG GAA AAA TAC CAA ACC AAG
7861 AAT AGA GAA GTT CAG ATC AAG GGC GGG TAC ATG AAA ATA GCT AAC GTT GGG CCA AAC AGG
7921 ATA TCT GCG GTG AGC AGT TTC GGC CCC GGC CCG GGG CCA AGA ACA GAT GGT CAC CGC AGT
7981 TTC GGC CCC GGC CCG AGG CCA AGA ACA GAT GGT CCC CAG ATA TGG CCC AAC CCT CAG CAG
8041 TTT CTT AAG ACC CAT CAG ATG TTT CCA GGC TCC CCC AAG GAC CTG AAA TGA CCC TGC GCC
8101 TTA TTT GAA TTA ACC AAT CAG CCT GCT TCT CGC TTC TGT TCG CGC GCT TCT GCT TCC CGA
8161 GCT CTA TAA AAG AGC TCA CAA CCC CTC ACT CGG CGC GCC AGT CCT CCG ACA GAC TGA GTC
8221 GCC CGG GGG GGA TCC GTC CTC CGG CCT CGG CTG CGT CGC GCC ATG GCG GCC CCC GGC GCC
                                                        M   A   A   P   G   A
8281 CGG CGG CCG CTG CTC CTG CTG CTG CTG GCA GGC CTT GCA CAT GGC GCC TCA GCA CTC TTT
      R   R   P   L   L   L   L   L   L   A   G   L   A   H   G   A   S   A   L   F
8341 GAG AAT TCG ATG GTC AGC AAG GGC GAG GAG CTG TTC ACC GGG GTG GTG CCC ATC CTG GTC
      E   N   S   M   V   S   K   G   E   E   L   F   T   G   V   V   P   I   L   V
8401 GAG CTG GAC GGC GAC GTA AAC GGC CAC AAG TTC AGC GTG TCC GGC GAG GGC GAG GGC GAT
      E   L   D   G   D   V   N   G   H   K   F   S   V   S   G   E   G   E   G   D
8461 GCC ACC TAC GGC AAG CTG ACC CTG AAG TTC ATC TGC ACC ACC GGC AAG CTG CCC GTG CCC
      A   T   Y   G   K   L   T   L   K   F   I   C   T   T   G   K   L   P   V   P
8521 TGG CCC ACC CTC GTG ACC ACC CTG ACC TAC GGC GTG CAG TGC TTC AGC CGC TAC CCC GAC
      W   P   T   L   V   T   T   L   T   Y   G   V   Q   C   F   S   R   Y   P   D
8581 CAC ATG AAG CAG CAC GAC TTC TTC AAG TCC GCC ATG CCC GAA GGC TAC GTC CAG GAG CGC
      H   M   K   Q   H   D   F   F   K   S   A   M   P   E   G   Y   V   Q   E   R
8641 ACC ATC TTC TTC AAG GAC GAC GGC AAC TAC AAG ACC CGC GCC GAG GTG AAG TTC GAG GGC
      T   I   F   F   K   D   D   G   N   Y   K   T   R   A   E   V   K   F   E   G
8701 GAC ACC CTG GTG AAC CGC ATC GAG CTG AAG GGC ATC GAC TTC AAG GAG GAC GGC AAC ATC
      D   T   L   V   N   R   I   E   L   K   G   I   D   F   K   E   D   G   N   I
8761 CTG GGG CAC AAG CTG GAG TAC AAC TAC AAC AGC CAC AAC GTC TAT ATC ATG GCC GAC AAG
      L   G   H   K   L   E   Y   N   Y   N   S   H   N   V   Y   I   M   A   D   K
8821 CAG AAG AAC GGC ATC AAG GTG AAC TTC AAG ATC CGC CAC AAC ATC GAG GAC GGC AGC GTG
      Q   K   N   G   I   K   V   N   F   K   I   R   H   N   I   E   D   G   S   V
8881 CAG CTC GCC GAC CAC TAC CAG CAG AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG CCC
      Q   L   A   D   H   Y   Q   Q   N   T   P   I   G   D   G   P   V   L   L   P
8941 GAC AAC CAC TAC CTG AGC ACC CAG TCC GCC CTG AGC AAA GAC CCC AAC GAG AAG CGC GAT
      D   N   H   Y   L   S   T   Q   S   A   L   S   K   D   P   N   E   K   R   D
9001 CAC ATG GTC CTG CTG GAG TTC GTG ACC GCC GCC GGG ATC ACT CTC GGC ATG GAC GAG CTG
      H   M   V   L   L   E   F   V   T   A   A   G   I   T   L   G   M   D   E   L
9061 TAC AAG CTT CGC ATG AAG GGA TCC GGC ATC GCT GGC TTC AAA GGT GAA CAA GGC CCC AAG
      Y   K   L   R   M   K   G   S   G   I   A   G   F   K   G   E   Q   G   P   K
9121 GGA GAA CCT TCA TGG GAG AAT CTG AAG AGC GGG ACC TCT GAG CCC ATC CGC GGG GAC TGG
      G   E   P   S   W   E   N   L   K   S   G   T   S   E   P   I   R   G   D   W
9181 ACA CCT GGG CTG TCC CCC ATC CAG ACA GTG AAG GTC TCT GTG TCT GCA GCC ACC CTG GGC
      T   P   G   L   S   P   I   Q   T   V   K   V   S   V   S   A   A   T   L   G
9241 CTG GGC TTC ATC ATC TTC TGT GTT GGC TTC TTC AGA TGG CGC AAG TCT CAT TCC TCC AGC
      L   G   F   I   I   F   C   V   G   F   F   R   W   R   K   S   H   S   S   S
9301 TAC ACT CCT CTC CCC GGA TCC ACC TAC CCA GAA GGA CGG CAC TAG CTC GAG AGG CCT GGT
      Y   T   P   L   P   G   S   T   Y   P   E   G   R   H   Z
9361 ACC ACG CGT GCG GCC GCG ACT CTA GAG TCG ACC TGC AGG CAT GCA AGC TTG ATA TCA AGC
9421 TTA TCG ATG AAT GTC AAT AAA ACA AAA TGG TGG GGC AAT CAT CTA CAT TTC ATG GGA TAT
9481 GTG ATT ACT AGT TCA GGT GTA TTG CCA CAA GAC AAA CAT GTT AAG AAA ATT TCC CGT TAT
9541 TTG CAC TCT GTT CCT GTT AAT CAA CCT CTG GAT TAC AAA ATT TGT GAA AGA TTG ACT GGT
9601 ATT CTT AAC TAT GTT GCT CCT TTT ACG CTA TGT GGA TAC GCT GCT TTA ATG CCT TTG TAT
9661 CAT GCT ATT GCT TCC CGT ATG GCT TTC ATT TTC TCC TCC TTG TAT AAA TCC TGG TTG CTG
9721 TCT CTT TAT GAG GAG TTG TGG CCC GTT GTC AGG CAA CGT GGC GTG GTG TGC ACT GTG TTT
9781 GCT GAC GCA ACC CCC ACT GGT TGG GGC ATT GCC ACC ACC TGT CAG CTC CTT TCC GGG ACT
9841 TTC GCT TTC CCC CTC CCT ATT GCC ACG GCG GAA CTC ATC GCC GCC TGC CTT GCC CGC TGC
9901 TGG ACA GGG GCT CGG CTG TTG GGC ACT GAC AAT TCC GTG GTG TTG TCG GGG AAG CTG ACG
9961 TCC TTT CCA TGG CTG CTC GCC TGT GTT GCC ACC TGG ATT CTG CGC GGG ACG TCC TTC TGC
10021 TAC GTC CCT TCG GCC CTC AAT CCA GCG GAC CTT CCT TCC CGC GGC CTG CTG CCG GCT CTG
10081 CGG CCT CTT CCG CGT CTT CGC CTT CGC CCT CAG ACG AGT CGG ATC TCC CTT TGG GCC GCC
10141 TCC CCG CCT GAT CGA TAC CGT CGA CCT CGA
```

DNA Sequence Translated - DNADynamo File pLssGCL12

1 CTT TAA GAC CAA TGA CTT ACA AGG CAG CTG TAG ATC TTA GCC ACT TTT TAA AAG AAA AGG
61 GGG GAC TGG AAG GGC TAA TTC ACT CCC AAC GAA GAC AAG ATC TGC TTT TTG CTT GTA CTG
121 GGT CTC TCT GGT TAG ACC AGA TCT GAG CCT GGG AGC TCT CTG GCT AAC TAG GGA ACC CAC
181 TGC TTA AGC CTC AAT AAA GCT TGC CTT GAG TGC TTC AAG TAG TGT GTG CCC GTC TGT TGT
241 GTG ACT CTG GTA ACT AGA GAT CCC TCA GAC CCT TTT AGT CAG TGT GGA AAA TCT CTA GCA
301 GCA TCT AGA ATT AAT TCC GTG TAT TCT ATA GTG TCA CCT AAA TCG TAT GTG TAT GAT ACA
361 TAA GGT TAT GTA TTA ATT GTA GCC GCG TTC TAA CGA CAA TAT GTA CAA GCC TAA TTG TGT
421 AGC ATC TGG CTT ACT GAA GCA GAC CCT ATC ATC TCT CTC GTA AAC TGC CGT CAG AGT CGG
481 TTT GGT TGG ACG AAC CTT CTG AGT TTC TGG TAA CGC CGT CCC GCA CCC GGA AAT GGT CAG
541 CGA ACC AAT CAG CAG GGT CAT CGC TAG CCA GAT CCT CTA CGC CGG ACG CAT CGT GGC CGG
601 CAT CAC CGG CGC CAC AGG TGC GGT TGC TGG CGC CTA TAT CGC CGA CAT CAC CGA TGG GGA
661 AGA TCG GGC TCG CCA CTT CGG GCT CAT GAG CGC TTG TTT CGG CGT GGG TAT GGT GGC AGG
721 CCC CGT GGC CGG GGG ACT GTT GGG CGC CAT CTC CTT GCA TGC ACC ATT CCT TGC GGC GGC
781 GGT GCT CAA CGG CCT CAA CCT ACT ACT GGG CTG CTT CCT AAT GCA GGA GTC GCA TAA GGG
841 AGA GCG TCG AAT GGT GCA CTC TCA GTA CAA TCT GCT CTG ATG CCG CAT AGT TAA GCC AGC
901 CCC GAC ACC CGC CAA CAC CCG CTG ACG CGC CCT GAC GGG CTT GTC TGC TCC CGG CAT CCG
961 CTT ACA GAC AAG CTG TGA CCG TCT CCG GGA GCT GCA TGT GTC AGA GGT TTT CAC CGT CAT
1021 CAC CGA AAC GCG CGA GAC GAA AGG GCC TCG TGA TAC GCC TAT TTT TAT AGG TTA ATG TCA
1081 TGA TAA TAA TGG TTT CTT AGA CGT CAG GTG GCA CTT TTC GGG GAA ATG TGC GCG GAA CCC
1141 CTA TTT GTT TAT TTT TCT AAA TAC ATT CAA ATA TGT ATC CGC TCA TGA GAC AAT AAC CCT
1201 GAT AAA TGC TTC AAT AAT ATT GAA AAA GGA AGA GTA TGA GTA TTC AAC ATT TCC GTG TCG
1261 CCC TTA TTC CCT TTT TTG CGG CAT TTT GCC TTC CTG TTT TTG CTC ACC CAG AAA CGC TGG
1321 TGA AAG TAA AAG ATG CTG AAG ATC AGT TGG GTG CAC GAG TGG GTT ACA TCG AAC TGG ATC
1381 TCA ACA GCG GTA AGA TCC TTG AGA GTT TTC GCC CCG AAG AAC GTT TTC CAA TGA TGA GCA
1441 CTT TTA AAG TTC TGC TAT GTG GCG CGG TAT TAT CCC GTA TTG ACG CCG GGC AAG AGC AAC
1501 TCG GTC GCC GCA TAC ACT ATT CTC AGA ATG ACT TGG TTG AGT ACT CAC CAG TCA CAG AAA
1561 AGC ATC TTA CGG ATG GCA TGA CAG TAA GAG AAT TAT GCA GTG CTG CCA TAA CCA TGA GTG
1621 ATA ACA CTG CGG CCA ACT TAC TTC TGA CAA CGA TCG GAG GAC CGA AGG AGC TAA CCG CTT
1681 TTT TGC ACA ACA TGG GGG ATC ATG TAA CTC GCC TTG ATC GTT GGG AAC CGG AGC TGA ATG
1741 AAG CCA TAC CAA ACG ACG AGC GTG ACA CCA CGA TGC CTG TAG CAA TGG CAA CAA CGT TGC
1801 GCA AAC TAT TAA CTG GCG AAC TAC TTA CTC TAG CTT CCC GGC AAC AAT TAA TAG ACT GGA
1861 TGG AGG CGG ATA AAG TTG CAG GAC CAC TTC TGC GCT CGG CCC TTC CGG CTG GCT GGT TTA
1921 TTG CTG ATA AAT CTG GAG CCG GTG AGC GTG GGT CTC GCG GTA TCA TTG CAG CAC TGG GGC
1981 CAG ATG GTA AGC CCT CCC GTA TCG TAG TTA TCT ACA CGA CGG GGA GTC AGG CAA CTA TGG
2041 ATG AAC GAA ATA GAC AGA TCG CTG AGA TAG GTG CCT CAC TGA TTA AGC ATT GGT AAC TGT
2101 CAG ACC AAG TTT ACT CAT ATA TAC TTT AGA TTG ATT TAA AAC TTC ATT TTT AAT TTA AAA
2161 GGA TCT AGG TGA AGA TCC TTT TTG ATA ATC TCA TGA CCA AAA TCC CTT AAC GTG AGT TTT
2221 CGT TCC ACT GAG CGT CAG ACC CCG TAG AAA AGA TCA AAG GAT CTT CTT GAG ATC CTT TTT
2281 TTC TGC GCG TAA TCT GCT GCT TGC AAA CAA AAA AAC CAC CGC TAC CAG CGG TGG TTT GTT
2341 TGC CGG ATC AAG AGC TAC CAA CTC TTT TTC CGA AGG TAA CTG GCT TCA GCA GAG CGC AGA
2401 TAC CAA ATA CTG TCC TTC TAG TGT AGC CGT AGT TAG GCC ACC ACT TCA AGA ACT CTG TAG
2461 CAC CGC CTA CAT ACC TCG CTC TGC TAA TCC TGT TAC CAG TGG CTG CTG CCA GTG GCG ATA
2521 AGT CGT GTC TTA CCG GGT TGG ACT CAA GAC GAT AGT TAC CGG ATA AGG CGC AGC GGT CGG
2581 GCT GAA CGG GGG GTT CGT GCA CAC AGC CCA GCT TGG AGC GAA CGA CCT ACA CCG AAC TGA
2641 GAT ACC TAC AGC GTG AGC ATT GAG AAA GCG CCA CGC TTC CCG AAG GGA GAA AGG CGG ACA
2701 GGT ATC CGG TAA GCG GCA GGG TCG GAA CAG GAG AGC GCA CGA GGG AGC TTC CAG GGG GAA
2761 ACG CCT GGT ATC TTT ATA GTC CTG TCG GGT TTC GCC ACC TCT GAC TTG AGC GTC GAT TTT
2821 TGT GAT GCT CGT CAG GGG GGC GGA GCC TAT GGA AAA ACG CCA GCA ACG CGG CCT TTT TAC
2881 GGT TCC TGG CCT TTT GCT GGC CTT TTG CTC ACA TGT TCT TTC CTG CGT TAT CCC CTG ATT
2941 CTG TGG ATA ACC GTA TTA CCG CCT TTG AGT GAG CTG ATA CCG CTC GCC GCA GCC GAA CGA
3001 CCG AGC GCA GCG AGT CAG TGA GCG AGG AAG CGG AAG AGC GCC CAA TAC GCA AAC CGC CTC
3061 TCC CCG CGC GTT GGC CGA TTC ATT AAT GCA GCT GTG GAA TGT GTG TCA GTT AGG GTG TGG
3121 AAA GTC CCC AGG CTC CCC AGC AGG CAG AAG TAT GCA AAG CAT GCA TCT CAA TTA GTC AGC
3181 AAC CAG GTG TGG AAA GTC CCC AGG CTC CCC AGC AGG CAG AAG TAT GCA AAG CAT GCA TCT
3241 CAA TTA GTC AGC AAC CAT AGT CCC GCC CCT AAC TCC GCC CAT CCC GCC CCT AAC TCC GCC
3301 CAG TTC CGC CCA TTC TCC GCC CCA TGG CTG ACT AAT TTT TTT TAT TTA TGC AGA GGC CGA
3361 GGC CGC CTC GGC CTC TGA GCT ATT CCA GAA GTA GTG AGG AGG CTT TTT TGG AGG CCT AGG
3421 CTT TTG CAA AAA GCT TGG ACA CAA GAC AGG CTT GCG AGA TAT GTT TGA GAA TAC CAC TTT
3481 ATC CCG CGT CAG GGA GAG GCA GTG CGT AAA AAG ACG CGG ACT CAT GTG AAA TAC TGG TTT
3541 TTA GTG CGC CAG ATC TCT ATA ATC TCG CGC AAC CTA TTT TCC CCT CGA ACA CTT TTT AAG
3601 CCG TAG ATA AAC AGG CTG GGA CAC TTC ACA TGA GCG AAA AAT ACA TCG TCA CCT GGG ACA
3661 TGT TGC AGA TCC ATG CAC GTA AAC TCG CAA GCC GAC TGA TGC CTT CTG AAC AAT GGA AAG
3721 GCA TTA TTG CCG TAA GCC GTG GCG GTC TGT ACC GGG TGC GTT ACT GGC GCG TGA ACT GGG

3781 TAT TCG TCA TGT CGA TAC CGT TTG TAT TTC CAG CTA CGA TCA CGA CAA CCA GCG CGA GCT
3841 TAA AGT GCT GAA ACG CGC AGA AGG CGA TGG CGA AGG CTT CAT CGT TAT TGA TGA CCT GGT
3901 GGA TAC CGG TGG TAC TGC GGT TGC GAT TCG TGA AAT GTA TCC AAA AGC GCA CTT TGT CAC
3961 CAT CTT CGC AAA ACC GGC TGG TCG TCC GCT GGT TGA TGA CTA TGT TGT TGA TAT CCC GCA
4021 AGA TAC CTG GAT TGA ACA GCC GTG GGA TAT GGG CGT CGT ATT CGT CCC GCC AAT CTC CGG
4081 TCG CTA ATC TTT TCA ACG CCT GGC ACT GCC GGG CGT TGT TCT TTT TAA CTT CAG GCG GGT
4141 TAC AAT AGT TTC CAG TAA GTA TTC TGG AGG CTG CAT CCA TGA CAC AGG CAA ACC TGA GCG
4201 AAA CCC TGT TCA AAC CCC GCT TTA AAC ATC CTG AAA CCT CGA CGC TAG TCC GCC GCT TTA
4261 ATC ACG GCG CAC AAC CGC CTG TGC AGT CGG CCC TTG ATG GTA AAA CCA TCC CTC ACT GGT
4321 ATC GCA TGA TTA ACC GTC TGA TGT GGA TCT GGC GCG GCA TTG ACC CAC GCG AAA TCC TCG
4381 ACG TCC AGG CAC GTA TTG TGA TGA GCG ATG CCG AAC GTA CCG ACG ATG ATT TAT ACG ATA
4441 CGG TGA TTG GCT ACC GTG GCG GCA ACT GGA TTT ATG AGT GGG CCC CGG ATC TTT GTG AAG
4501 GAA CCT TAC TTC TGT GGT GTG ACA TAA TTG GAC AAA CTA CCT ACA GAG ATT TAA AGC TCT
4561 AAG GTA AAT ATA AAA TTT TTA AGT GTA TAA TGT GTT AAA CTA CTG ATT CTA ATT GTT TGT
4621 GTA TTT TAG ATT CCA ACC TAT GGA ACT GAT GAA TGG GAG CAG TGG TGG AAT GCC TTT AAT
4681 GAG GAA AAC CTG TTT TGC TCA GAA GAA ATG CCA TCT AGT GAT GAT GAG GCT ACT GCT GAC
4741 TCT CAA CAT TCT ACT CCT CCA AAA AAG AAG AGA AAG GTA GAA GAC CCC AAG GAC TTT CCT
4801 TCA GAA TTG CTA AGT TTT TTG AGT CAT GCT GTG TTT AGT AAT AGA ACT CTT GCT TGC TTT
4861 GCT ATT TAC ACC ACA AAG GAA AAA GCT GCA CTG CTA TAC AAG AAA ATT ATG GAA AAA TAT
4921 TCT GTA ACC TTT ATA AGT AGG CAT AAC AGT TAT AAT CAT AAC ATA CTG TTT TTT CTT ACT
4981 CCA CAC AGG CAT AGA GTG TCT GCT ATT AAT AAC TAT GCT CAA AAA TTG TGT ACC TTT AGC
5041 TTT TTA ATT TGT AAA GGG GTT AAT AAG GAA TAT TTG ATG TAT AGT GCC TTG ACT AGA GAT
5101 CAT AAT CAG CCA TAC CAC ATT TGT AGA GGT TTT ACT TGC TTT AAA AAA CCT CCC ACA CCT
5161 CCC CCT GAA CCT GAA ACA TAA AAT GAA TGC AAT TGT TGT TGT TAA CTT GTT TAT TGC AGC
5221 TTA TAA TGG TTA CAA ATA AAG CAA TAG CAT CAC AAA TTT CAC AAA TAA AGC ATT TTT TTC
5281 ACT GCA TTC TAG TTG TGG TTT GTC CAA ACT CAT CAA TGT ATC TTA TCA TGT CTG GAT CAA
5341 CTG GAT AAC TCA AGC TAA CCA AAA TCA TCC CAA ACT TCC CAC CCC ATA CCC TAT TAC CAC
5401 TGC CAA TTA CCT AGT GGT TTC ATT TAC TCT AAA CCT GTG ATT CCT CTG AAT TAT TTT CAT
5461 TTT AAA GAA ATT GTA TTT GTT AAA TAT GTA CTA CAA ACT TAG TAG TTG GAA GGG CTA ATT
5521 CAC TCC CAA AGA AGA CAA GAT ATC CTT GAT CTG TGG ATC TAC CAC ACA CAA GGC TAC TTC
5581 CCT GAT TAG CAG AAC TAC ACA CCA GGG CCA GGG GTC AGA TAT CCA CTG ACC TTT GGA TGG
5641 TGC TAC AAG CTA GTA CCA GTT GAG CCA GAT AAG GTA GAA GAG GCC AAT AAA GGA GAG AAC
5701 ACC AGC TTG TTA CAC CCT GTG AGC CTG CAT GGG ATG GAT GAC CCG GAG AGA GAA GTG TTA
5761 GAG TGG AGG TTT GAC AGC CGC CTA GCA TTT CAT CAC GTG GCC CGA GAG CTG CAT CCG GAG
5821 TAC TTC AAG AAC TGC TGA TAT CGA GCT TGC TAC AAG GGA CTT TCC GCT GGG GAC TTT CCA
5881 GGG AGG CGT GGC CTG GGC GGG ACT GGG GAG TGG CGA GCC CTC AGA TCC TGC ATA TAA GCA
5941 GCT GCT TTT TGC CTG TAC TGG GTC TCT CTG GTT AGA CCA GAT CTG AGC CTG GGA GCT CTC
6001 TGG CTA ACT AGG GAA CCC ACT GCT TAA GCC TCA ATA AAG CTT GCC TTG AGT GCT TCA AGT
6061 AGT GTG TGC CCG TCT GTT GTG TGA CTC TGG TAA CTA GAG ATC CCT CAG ACC CTT TTA GTC
6121 AGT GTG GAA AAT CTC TAG CAG TGG CGC CCG AAC AGG GAC TTG AAA GCG AAA GGG AAA CCA
6181 GAG GAG CTC TCT CGA CGC AGG ACT CGG CTT GCT GAA GCG CGC ACG GCA AGA GGC GAG GGG
6241 CGG CGA CTG GTG AGT ACG CCA AAA ATT TTG ACT AGC GGA GGC TAG AAG GAG AGA GAT GGG
6301 TGC GAG AGC GTC AGT ATT AAG CGG GGG AGA ATT AGA TCG CGA TGG GAA AAA ATT CGG TTA
6361 AGG CCA GGG GGA AAG AAA AAA TAT AAA TTA AAA CAT ATA GTA TGG GCA AGC AGG GAG CTA
6421 GAA CGA TTC GCA GTT AAT CCT GGC CTG TTA GAA ACA TCA GAA GGC TGT AGA CAA ATA CTG
6481 GGA CAG CTA CAA CCA TCC CTT CAG ACA GGA TCA GAA GAA CTT AGA TCA TTA TAT AAT ACA
6541 GTA GCA ACC CTC TAT TGT GTG CAT CAA AGG ATA GAG ATA AAA GAC ACC AAG GAA GCT TTA
6601 GAC AAG ATA GAG GAA GAG CAA AAC AAA AGT AAG ACC ACC GCA CAG CAA GCG GCC GCT GAT
6661 CTT CAG ACC TGG AGG AGG AGA TAT GAG GGA CAA TTG GAG AAG TGA ATT ATA TAA ATA TAA
6721 AGT AGT AAA AAT TGA ACC ATT AGG AGT AGC ACC CAC CAA GGC AAA GAG AAG AGT GGT GCA
6781 GAG AGA AAA AAG AGC AGT GGG AAT AGG AGC TTT GTT CCT TGG GTT CTT GGG AGC AGC AGG
6841 AAG CAC TAT GGG CGC AGC GTC AAT GAC GCT GAC GGT ACA GGC CAG ACA ATT ATT GTC TGG
6901 TAT AGT GCA GCA GCA GAA CAA TTT GCT GAG GGC TAT TGA GGC GCA ACA GCA TCT GTT GCA
6961 ACT CAC AGT CTG GGG CAT CAA GCA GCT CCA GGC AAG AAT CCT GGC TGT GGA AAG ATA CCT
7021 AAA GGA TCA ACA GCT CCT GGG GAT TTG GGG TTG CTC TGG AAA ACT CAT TTG CAC CAC TGC
7081 TGT GCC TTG GAA TGC TAG TTG GAG TAA TAA ATC TCT GGA ACA GAT TTG GAA TCA CAC GAC
7141 CTG GAT GGA GTG GGA CAG AGA AAT TAA CAA TTA CAC AAG CTT AAT ACA CTC CTT AAT TGA
7201 AGA ATC GCA AAA CCA GCA AGA AAA GAA TGA ACA AGA ATT ATT GGA ATT AGA TAA ATG GGC
7261 AAG TTT GTG GAA TTG GTT TAA CAT AAC AAA TTG GCT GTG GTA TAT AAA ATT ATT CAT AAT
7321 GAT AGT AGG AGG CTT GGT AGG TTT AAG AAT AGT TTT TGC TGT ACT TTC TAT AGT GAA TAG
7381 AGT TAG GCA GGG ATA TTC ACC ATT ATC GTT TCA GAC CCA CCT CCC AAC CCC GAG GGG ACC
7441 CGA CAG GCC CGA AGG AAT AGA AGA AGA AGG TGG AGA GAG AGA CAG AGA CAG ATC CAT TCG
7501 ATT AGT GAA CGG ATC TCG ACG GTC GCC AAA TGG CAG TAT TCA TCC ACA ATT TTA AAA GAA
7561 AAG GGG GGA TTG GGG GGT ACA GTG CAG GGG AAA GAA TAG TAG ACA TAA TAG CAA CAG ACA

```
7621 TAC AAA CTA AAG AAT TAC AAA AAC AAA TTA CAA AAA TTC AAA ATT TTC GGG TTT ATT ACA
7681 GGG ACA GCA GAG ATC CAG TTT GGA TCG ATA AGC TTG ATA TCG AAT TCC TGC AGC CCC GAT
7741 AAA ATA AAA GAT TTT ATT TAG TCT CCA GAA AAA GGG GGG AAT GAA AGA CCC CAC CTG TAG
7801 GTT TGG CAA GCT AGC TGC AGT AAC GCC ATT TTG CAA GGC ATG GAA AAA TAC CAA ACC AAG
7861 AAT AGA GAA GTT CAG ATC AAG GGC GGG TAC ATG AAA ATA GCT AAC GTT GGG CCA AAC AGG
7921 ATA TCT GCG GTG AGC AGT TTC GGC CCC GGC CCG GGG CCA AGA ACA GAT GGT CAC CGC AGT
7981 TTC GGC CCC GGC CCG AGG CCA AGA ACA GAT GGT CCC CAG ATA TGG CCC AAC CCT CAG CAG
8041 TTT CTT AAG ACC CAT CAG ATG TTT CCA GGC TCC CCC AAG GAC CTG AAA TGA CCC TGC GCC
8101 TTA TTT GAA TTA ACC AAT CAG CCT GCT TCT CGC TTC TGT TCG CGC GCT TCT GCT TCC CGA
8161 GCT CTA TAA AAG AGC TCA CAA CCC CTC ACT CGG CGC GCC AGT CCT CCG ACA GAC TGA GTC
8221 GCC CGG GGG GGA TCC GTC CTC CGG CCT CGG CTG CGT CGC GCC ATG GCG GCC CCC GGC GCC
                                                          M   A   A   P   G   A
8281 CGG CGG CCG CTG CTC CTG CTG CTG CTG GCA GGC CTT GCA CAT GGC GCC TCA GCA CTC TTT
      R   R   P   L   L   L   L   L   L   A   G   L   A   H   G   A   S   A   L   F
8341 GAG AAT TCG ATG GTC AGC AAG GGC GAG GAG CTG TTC ACC GGG GTG GTG CCC ATC CTG GTC
      E   N   S   M   V   S   K   G   E   E   L   F   T   G   V   V   P   I   L   V
8401 GAG CTG GAC GGC GAC GTA AAC GGC CAC AAG TTC AGC GTG TCC GGC GAG GGC GAG GGC GAT
      E   L   D   G   D   V   N   G   H   K   F   S   V   S   G   E   G   E   G   D
8461 GCC ACC TAC GGC AAG CTG ACC CTG AAG TTC ATC TGC ACC ACC GGC AAG CTG CCC GTG CCC
      A   T   Y   G   K   L   T   L   K   F   I   C   T   T   G   K   L   P   V   P
8521 TGG CCC ACC CTC GTG ACC ACC CTG ACC TAC GGC GTG CAG TGC TTC AGC CGC TAC CCC GAC
      W   P   T   L   V   T   T   L   T   Y   G   V   Q   C   F   S   R   Y   P   D
8581 CAC ATG AAG CAG CAC GAC TTC TTC AAG TCC GCC ATG CCC GAA GGC TAC GTC CAG GAG CGC
      H   M   K   Q   H   D   F   F   K   S   A   M   P   E   G   Y   V   Q   E   R
8641 ACC ATC TTC TTC AAG GAC GAC GGC AAC TAC AAG ACC CGC GCC GAG GTG AAG TTC GAG GGC
      T   I   F   F   K   D   D   G   N   Y   K   T   R   A   E   V   K   F   E   G
8701 GAC ACC CTG GTG AAC CGC ATC GAG CTG AAG GGC ATC GAC TTC AAG GAG GAC GGC AAC ATC
      D   T   L   V   N   R   I   E   L   K   G   I   D   F   K   E   D   G   N   I
8761 CTG GGG CAC AAG CTG GAG TAC AAC TAC AAC AGC CAC AAC GTC TAT ATC ATG GCC GAC AAG
      L   G   H   K   L   E   Y   N   Y   N   S   H   N   V   Y   I   M   A   D   K
8821 CAG AAG AAC GGC ATC AAG GTG AAC TTC AAG ATC CGC CAC AAC ATC GAG GAC GGC AGC GTG
      Q   K   N   G   I   K   V   N   F   K   I   R   H   N   I   E   D   G   S   V
8881 CAG CTC GCC GAC CAC TAC CAG CAG AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG CCC
      Q   L   A   D   H   Y   Q   Q   N   T   P   I   G   D   G   P   V   L   L   P
8941 GAC AAC CAC TAC CTG AGC ACC CAG TCC GCC CTG AGC AAA GAC CCC AAC GAG AAG CGC GAT
      D   N   H   Y   L   S   T   Q   S   A   L   S   K   D   P   N   E   K   R   D
9001 CAC ATG GTC CTG CTG GAG TTC GTG ACC GCC GCC GGG ATC ACT CTC GGC ATG GAC GAG CTG
      H   M   V   L   L   E   F   V   T   A   A   G   I   T   L   G   M   D   E   L
9061 TAC AAG CTT CGC ATG AAG GGA TCC GGC ATC GCT GGC TTC AAA GGT GAA CAA GGC CCC AAG
      Y   K   L   R   M   K   G   S   G   I   A   G   F   K   G   E   Q   G   P   K
9121 GGA GAA CCT TCA TGG GAG AAT CTG AAG GGG TCT GTG GAA GAG TGT GTT CAG GAT GGT AAC
      G   E   P   S   W   E   N   L   K   G   S   V   E   E   C   V   Q   D   G   N
9181 AAC ATG TTG ATC CCC ATT GCT GTG GGC GGT GCC CTG GCA GGG CTG GTC CTC ATC GTC CTC
      N   M   L   I   P   I   A   V   G   G   A   L   A   G   L   V   L   I   V   L
9241 ATT GCC TAC CTC ATT GGC AGG AAG AGG AGT CAC GCC GGC TAT CAG ACC ATC TAG CTC GAG
      I   A   Y   L   I   G   R   K   R   S   H   A   G   Y   Q   T   I   Z
9301 AGG CCT GGT ACC ACG CGT GCG GCC GCG ACT CTA GAG TCG ACC TGC AGG CAT GCA AGC TTG
9361 ATA TCA AGC TTA TCG ATG AAT GTC AAT AAA ACA AAA TGG TGG GGC AAT CAT CTA CAT TTC
9421 ATG GGA TAT GTG ATT ACT AGT TCA GGT GTA TTG CCA CAA GAC AAA CAT GTT AAG AAA ATT
9481 TCC CGT TAT TTG CAC TCT GTT CCT GTT AAT CAA CCT CTG GAT TAC AAA ATT TGT GAA AGA
9541 TTG ACT GGT ATT CTT AAC TAT GTT GCT CCT TTT ACG CTA TGT GGA TAC GCT GCT TTA ATG
9601 CCT TTG TAT CAT GCT ATT GCT TCC CGT ATG GCT TTC ATT TTC TCC TCC TTG TAT AAA TCC
9661 TGG TTG CTG TCT CTT TAT GAG GAG TTG TGG CCC GTT GTC AGG CAA CGT GGC GTG GTG TGC
9721 ACT GTG TTT GCT GAC GCA ACC CCC ACT GGT TGG GGC ATT GCC ACC ACC TGT CAG CTC CTT
9781 TCC GGG ACT TTC GCT TTC CCC CTC CCT ATT GCC ACG GCG GAA CTC ATC GCC GCC TGC CTT
9841 GCC CGC TGC TGG ACA GGG GCT CGG CTG TTG GGC ACT GAC AAT TCC GTG GTG TTG TCG GGG
9901 AAG CTG ACG TCC TTT CCA TGG CTG CTC GCC TGT GTT GCC ACC TGG ATT CTG CGC GGG ACG
9961 TCC TTC TGC TAC GTC CCT TCG GCC CTC AAT CCA GCG GAC CTT CCT TCC CGC GGC CTG CTG
10021 CCG GCT CTG CGG CCT CTT CCG CGT CTT CGC CTT CGC CCT CAG ACG AGT CGG ATC TCC CTT
10081 TGG GCC GCC TCC CCG CCT GAT CGA TAC CGT CGA CCT CGA
```

DNA Sequence Translated - DNADynamo File pMOG-LAMP

1 CTT TAA GAC CAA TGA CTT ACA AGG CAG CTG TAG ATC TTA GCC ACT TTT TAA AAG AAA AGG
61 GGG GAC TGG AAG GGC TAA TTC ACT CCC AAC GAA GAC AAG ATC TGC TTT TTG CTT GTA CTG
121 GGT CTC TCT GGT TAG ACC AGA TCT GAG CCT GGG AGC TCT CTG GCT AAC TAG GGA ACC CAC
181 TGC TTA AGC CTC AAT AAA GCT TGC CTT GAG TGC TTC AAG TAG TGT GTG CCC GTC TGT TGT
241 GTG ACT CTG GTA ACT AGA GAT CCC TCA GAC CCT TTT AGT CAG TGT GGA AAA TCT CTA GCA
301 GCA TCT AGA ATT AAT TCC GTG TAT TCT ATA GTG TCA CCT AAA TCG TAT GTG TAT GAT ACA
361 TAA GGT TAT GTA TTA ATT GTA GCC GCG TTC TAA CGA CAA TAT GTA CAA GCC TAA TTG TGT
421 AGC ATC TGG CTT ACT GAA GCA GAC CCT ATC ATC TCT CTC GTA AAC TGC CGT CAG AGT CGG
481 TTT GGT TGG ACG AAC CTT CTG AGT TTC TGG TAA CGC CGT CCC GCA CCC GGA AAT GGT CAG
541 CGA ACC AAT CAG CAG GGT CAT CGC TAG CCA GAT CCT CTA CGC CGG ACG CAT CGT GGC CGG
601 CAT CAC CGG CGC CAC AGG TGC GGT TGC TGG CGC CTA TAT CGC CGA CAT CAC CGA TGG GGA
661 AGA TCG GGC TCG CCA CTT CGG GCT CAT GAG CGC TTG TTT CGG CGT GGG TAT GGT GGC AGG
721 CCC CGT GGC CGG GGG ACT GTT GGG CGC CAT CTC CTT GCA TGC ACC ATT CCT TGC GGC GGC
781 GGT GCT CAA CGG CCT CAA CCT ACT ACT GGG CTG CTT CCT AAT GCA GGA GTC GCA TAA GGG
841 AGA GCG TCG AAT GGT GCA CTC TCA GTA CAA TCT GCT CTG ATG CCG CAT AGT TAA GCC AGC
901 CCC GAC ACC CGC CAA CAC CCG CTG ACG CGC CCT GAC GGG CTT GTC TGC TCC CGG CAT CCG
961 CTT ACA GAC AAG CTG TGA CCG TCT CCG GGA GCT GCA TGT GTC AGA GGT TTT CAC CGT CAT
1021 CAC CGA AAC GCG CGA GAC GAA AGG GCC TCG TGA TAC GCC TAT TTT TAT AGG TTA ATG TCA
1081 TGA TAA TAA TGG TTT CTT AGA CGT CAG GTG GCA CTT TTC GGG GAA ATG TGC GCG GAA CCC
1141 CTA TTT GTT TAT TTT TCT AAA TAC ATT CAA ATA TGT ATC CGC TCA TGA GAC AAT AAC CCT
1201 GAT AAA TGC TTC AAT AAT ATT GAA AAA GGA AGA GTA TGA GTA TTC AAC ATT TCC GTG TCG
1261 CCC TTA TTC CCT TTT TTG CGG CAT TTT GCC TTC CTG TTT TTG CTC ACC CAG AAA CGC TGG
1321 TGA AAG TAA AAG ATG CTG AAG ATC AGT TGG GTG CAC GAG TGG GTT ACA TCG AAC TGG ATC
1381 TCA ACA GCG GTA AGA TCC TTG AGA GTT TTC GCC CCG AAG AAC GTT TTC CAA TGA TGA GCA
1441 CTT TTA AAG TTC TGC TAT GTG GCG CGG TAT TAT CCC GTA TTG ACG CCG GGC AAG AGC AAC
1501 TCG GTC GCC GCA TAC ACT ATT CTC AGA ATG ACT TGG TTG AGT ACT CAC CAG TCA CAG AAA
1561 AGC ATC TTA CGG ATG GCA TGA CAG TAA GAG AAT TAT GCA GTG CTG CCA TAA CCA TGA GTG
1621 ATA ACA CTG CGG CCA ACT TAC TTC TGA CAA CGA TCG GAG GAC CGA AGG AGC TAA CCG CTT
1681 TTT TGC ACA ACA TGG GGG ATC ATG TAA CTC GCC TTG ATC GTT GGG AAC CGG AGC TGA ATG
1741 AAG CCA TAC CAA ACG ACG AGC GTG ACA CCA CGA TGC CTG TAG CAA TGG CAA CAA CGT TGC
1801 GCA AAC TAT TAA CTG GCG AAC TAC TTA CTC TAG CTT CCC GGC AAC AAT TAA TAG ACT GGA
1861 TGG AGG CGG ATA AAG TTG CAG GAC CAC TTC TGC GCT CGG CCC TTC CGG CTG GCT GGT TTA
1921 TTG CTG ATA AAT CTG GAG CCG GTG AGC GTG GGT CTC GCG GTA TCA TTG CAG CAC TGG GGC
1981 CAG ATG GTA AGC CCT CCC GTA TCG TAG TTA TCT ACA CGA CGG GGA GTC AGG CAA CTA TGG
2041 ATG AAC GAA ATA GAC AGA TCG CTG AGA TAG GTG CCT CAC TGA TTA AGC ATT GGT AAC TGT
2101 CAG ACC AAG TTT ACT CAT ATA TAC TTT AGA TTG ATT TAA AAC TTC ATT TTT AAT TTA AAA
2161 GGA TCT AGG TGA AGA TCC TTT TTG ATA ATC TCA TGA CCA AAA TCC CTT AAC GTG AGT TTT
2221 CGT TCC ACT GAG CGT CAG ACC CCG TAG AAA AGA TCA AAG GAT CTT CTT GAG ATC CTT TTT
2281 TTC TGC GCG TAA TCT GCT GCT TGC AAA CAA AAA AAC CAC CGC TAC CAG CGG TGG TTT GTT
2341 TGC CGG ATC AAG AGC TAC CAA CTC TTT TTC CGA AGG TAA CTG GCT TCA GCA GAG CGC AGA
2401 TAC CAA ATA CTG TCC TTC TAG TGT AGC CGT AGT TAG GCC ACC ACT TCA AGA ACT CTG TAG
2461 CAC CGC CTA CAT ACC TCG CTC TGC TAA TCC TGT TAC CAG TGG CTG CTG CCA GTG GCG ATA
2521 AGT CGT GTC TTA CCG GGT TGG ACT CAA GAC GAT AGT TAC CGG ATA AGG CGC AGC GGT CGG
2581 GCT GAA CGG GGG GTT CGT GCA CAC AGC CCA GCT TGG AGC GAA CGA CCT ACA CCG AAC TGA
2641 GAT ACC TAC AGC GTG AGC ATT GAG AAA GCG CCA CGC TTC CCG AAG GGA GAA AGG CGG ACA
2701 GGT ATC CGG TAA GCG GCA GGG TCG GAA CAG GAG AGC GCA CGA GGG AGC TTC CAG GGG GAA
2761 ACG CCT GGT ATC TTT ATA GTC CTG TCG GGT TTC GCC ACC TCT GAC TTG AGC GTC GAT TTT
2821 TGT GAT GCT CGT CAG GGG GGC GGA GCC TAT GGA AAA ACG CCA GCA ACG CGG CCT TTT TAC
2881 GGT TCC TGG CCT TTT GCT GGC CTT TTG CTC ACA TGT TCT TTC CTG CGT TAT CCC CTG ATT
2941 CTG TGG ATA ACC GTA TTA CCG CCT TTG AGT GAG CTG ATA CCG CTC GCC GCA GCC GAA CGA
3001 CCG AGC GCA GCG AGT CAG TGA GCG AGG AAG CGG AAG AGC GCC CAA TAC GCA AAC CGC CTC
3061 TCC CCG CGC GTT GGC CGA TTC ATT AAT GCA GCT GTG GAA TGT GTG TCA GTT AGG GTG TGG
3121 AAA GTC CCC AGG CTC CCC AGC AGG CAG AAG TAT GCA AAG CAT GCA TCT CAA TTA GTC AGC
3181 AAC CAG GTG TGG AAA GTC CCC AGG CTC CCC AGC AGG CAG AAG TAT GCA AAG CAT GCA TCT
3241 CAA TTA GTC AGC AAC CAT AGT CCC GCC CCT AAC TCC GCC CAT CCC GCC CCT AAC TCC GCC
3301 CAG TTC CGC CCA TTC TCC GCC CCA TGG CTG ACT AAT TTT TTT TAT TTA TGC AGA GGC CGA
3361 GGC CGC CTC GGC CTC TGA GCT ATT CCA GAA GTA GTG AGG AGG CTT TTT TGG AGG CCT AGG
3421 CTT TTG CAA AAA GCT TGG ACA CAA GAC AGG CTT GCG AGA TAT GTT TGA GAA TAC CAC TTT
3481 ATC CCG CGT CAG GGA GAG GCA GTG CGT AAA AAG ACG CGG ACT CAT GTG AAA TAC TGG TTT
3541 TTA GTG CGC CAG ATC TCT ATA ATC TCG CGC AAC CTA TTT TCC CCT CGA ACA CTT TTT AAG
3601 CCG TAG ATA AAC AGG CTG GGA CAC TTC ACA TGA GCG AAA AAT ACA TCG TCA CCT GGG ACA
3661 TGT TGC AGA TCC ATG CAC GTA AAC TCG CAA GCC GAC TGA TGC CTT CTG AAC AAT GGA AAG
3721 GCA TTA TTG CCG TAA GCC GTG GCG GTC TGT ACC GGG TGC GTT ACT GGC GCG TGA ACT GGG

3781 TAT TCG TCA TGT CGA TAC CGT TTG TAT TTC CAG CTA CGA TCA CGA CAA CCA GCG CGA GCT
3841 TAA AGT GCT GAA ACG CGC AGA AGG CGA TGG CGA AGG CTT CAT CGT TAT TGA TGA CCT GGT
3901 GGA TAC CGG TGG TAC TGC GGT TGC GAT TCG TGA AAT GTA TCC AAA AGC GCA CTT TGT CAC
3961 CAT CTT CGC AAA ACC GGC TGG TCG TCC GCT GGT TGA TGA CTA TGT TGT TGA TAT CCC GCA
4021 AGA TAC CTG GAT TGA ACA GCC GTG GGA TAT GGG CGT CGT ATT CGT CCC GCC AAT CTC CGG
4081 TCG CTA ATC TTT TCA ACG CCT GGC ACT GCC GGG CGT TGT TCT TTT TAA CTT CAG GCG GGT
4141 TAC AAT AGT TTC CAG TAA GTA TTC TGG AGG CTG CAT CCA TGA CAC AGG CAA ACC TGA GCG
4201 AAA CCC TGT TCA AAC CCC GCT TTA AAC ATC CTG AAA CCT CGA CGC TAG TCC GCC GCT TTA
4261 ATC ACG GCG CAC AAC CGC CTG TGC AGT CGG CCC TTG ATG GTA AAA CCA TCC CTC ACT GGT
4321 ATC GCA TGA TTA ACC GTC TGA TGT GGA TCT GGC GCG GCA TTG ACC CAC GCG AAA TCC TCG
4381 ACG TCC AGG CAC GTA TTG TGA TGA GCG ATG CCG AAC GTA CCG ACG ATG ATT TAT ACG ATA
4441 CGG TGA TTG GCT ACC GTG GCG GCA ACT GGA TTT ATG AGT GGG CCC CGG ATC TTT GTG AAG
4501 GAA CCT TAC TTC TGT GGT GTG ACA TAA TTG GAC AAA CTA CCT ACA GAG ATT TAA AGC TCT
4561 AAG GTA AAT ATA AAA TTT TTA AGT GTA TAA TGT GTT AAA CTA CTG ATT CTA ATT GTT TGT
4621 GTA TTT TAG ATT CCA ACC TAT GGA ACT GAT GAA TGG GAG CAG TGG TGG AAT GCC TTT AAT
4681 GAG GAA AAC CTG TTT TGC TCA GAA GAA ATG CCA TCT AGT GAT GAT GAG GCT ACT GCT GAC
4741 TCT CAA CAT TCT ACT CCT CCA AAA AAG AAG AGA AAG GTA GAA GAC CCC AAG GAC TTT CCT
4801 TCA GAA TTG CTA AGT TTT TTG AGT CAT GCT GTG TTT AGT AAT AGA ACT CTT GCT TGC TTT
4861 GCT ATT TAC ACC ACA AAG GAA AAA GCT GCA CTG CTA TAC AAG AAA ATT ATG GAA AAA TAT
4921 TCT GTA ACC TTT ATA AGT AGG CAT AAC AGT TAT AAT CAT AAC ATA CTG TTT TTT CTT ACT
4981 CCA CAC AGG CAT AGA GTG TCT GCT ATT AAT AAC TAT GCT CAA AAA TTG TGT ACC TTT AGC
5041 TTT TTA ATT TGT AAA GGG GTT AAT AAG GAA TAT TTG ATG TAT AGT GCC TTG ACT AGA GAT
5101 CAT AAT CAG CCA TAC CAC ATT TGT AGA GGT TTT ACT TGC TTT AAA AAA CCT CCC ACA CCT
5161 CCC CCT GAA CCT GAA ACA TAA AAT GAA TGC AAT TGT TGT TGT TAA CTT GTT TAT TGC AGC
5221 TTA TAA TGG TTA CAA ATA AAG CAA TAG CAT CAC AAA TTT CAC AAA TAA AGC ATT TTT TTC
5281 ACT GCA TTC TAG TTG TGG TTT GTC CAA ACT CAT CAA TGT ATC TTA TCA TGT CTG GAT CAA
5341 CTG GAT AAC TCA AGC TAA CCA AAA TCA TCC CAA ACT TCC CAC CCC ATA CCC TAT TAC CAC
5401 TGC CAA TTA CCT AGT GGT TTC ATT TAC TCT AAA CCT GTG ATT CCT CTG AAT TAT TTT CAT
5461 TTT AAA GAA ATT GTA TTT GTT AAA TAT GTA CTA CAA ACT TAG TAG TTG GAA GGG CTA ATT
5521 CAC TCC CAA AGA AGA CAA GAT ATC CTT GAT CTG TGG ATC TAC CAC ACA CAA GGC TAC TTC
5581 CCT GAT TAG CAG AAC TAC ACA CCA GGG CCA GGG GTC AGA TAT CCA CTG ACC TTT GGA TGG
5641 TGC TAC AAG CTA GTA CCA GTT GAG CCA GAT AAG GTA GAA GAG GCC AAT AAA GGA GAG AAC
5701 ACC AGC TTG TTA CAC CCT GTG AGC CTG CAT GGG ATG GAT GAC CCG GAG AGA GAA GTG TTA
5761 GAG TGG AGG TTT GAC AGC CGC CTA GCA TTT CAT CAC GTG GCC CGA GAG CTG CAT CCG GAG
5821 TAC TTC AAG AAC TGC TGA TAT CGA GCT TGC TAC AAG GGA CTT TCC GCT GGG GAC TTT CCA
5881 GGG AGG CGT GGC CTG GGC GGG ACT GGG GAG TGG CGA GCC CTC AGA TCC TGC ATA TAA GCA
5941 GCT GCT TTT TGC CTG TAC TGG GTC TCT CTG GTT AGA CCA GAT CTG AGC CTG GGA GCT CTC
6001 TGG CTA ACT AGG GAA CCC ACT GCT TAA GCC TCA ATA AAG CTT GCC TTG AGT GCT TCA AGT
6061 AGT GTG TGC CCG TCT GTT GTG TGA CTC TGG TAA CTA GAG ATC CCT CAG ACC CTT TTA GTC
6121 AGT GTG GAA AAT CTC TAG CAG TGG CGC CCG AAC AGG GAC TTG AAA GCG AAA GGG AAA CCA
6181 GAG GAG CTC TCT CGA CGC AGG ACT CGG CTT GCT GAA GCG CGC ACG GCA AGA GGC GAG GGG
6241 CGG CGA CTG GTG AGT ACG CCA AAA ATT TTG ACT AGC GGA GGC TAG AAG GAG AGA GAT GGG
6301 TGC GAG AGC GTC AGT ATT AAG CGG GGG AGA ATT AGA TCG CGA TGG GAA AAA ATT CGG TTA
6361 AGG CCA GGG GGA AAG AAA AAA TAT AAA TTA AAA CAT ATA GTA TGG GCA AGC AGG GAG CTA
6421 GAA CGA TTC GCA GTT AAT CCT GGC CTG TTA GAA ACA TCA GAA GGC TGT AGA CAA ATA CTG
6481 GGA CAG CTA CAA CCA TCC CTT CAG ACA GGA TCA GAA GAA CTT AGA TCA TTA TAT AAT ACA
6541 GTA GCA ACC CTC TAT TGT GTG CAT CAA AGG ATA GAG ATA AAA GAC ACC AAG GAA GCT TTA
6601 GAC AAG ATA GAG GAA GAG CAA AAC AAA AGT AAG ACC ACC GCA CAG CAA GCG GCC GCT GAT
6661 CTT CAG ACC TGG AGG AGG AGA TAT GAG GGA CAA TTG GAG AAG TGA ATT ATA TAA ATA TAA
6721 AGT AGT AAA AAT TGA ACC ATT AGG AGT AGC ACC CAC CAA GGC AAA GAG AAG AGT GGT GCA
6781 GAG AGA AAA AAG AGC AGT GGG AAT AGG AGC TTT GTT CCT TGG GTT CTT GGG AGC AGC AGG
6841 AAG CAC TAT GGG CGC AGC GTC AAT GAC GCT GAC GGT ACA GGC CAG ACA ATT ATT GTC TGG
6901 TAT AGT GCA GCA GCA GAA CAA TTT GCT GAG GGC TAT TGA GGC GCA ACA GCA TCT GTT GCA
6961 ACT CAC AGT CTG GGG CAT CAA GCA GCT CCA GGC AAG AAT CCT GGC TGT GGA AAG ATA CCT
7021 AAA GGA TCA ACA GCT CCT GGG GAT TTG GGG TTG CTC TGG AAA ACT CAT TTG CAC CAC TGC
7081 TGT GCC TTG GAA TGC TAG TTG GAG TAA TAA ATC TCT GGA ACA GAT TTG GAA TCA CAC GAC
7141 CTG GAT GGA GTG GGA CAG AGA AAT TAA CAA TTA CAC AAG CTT AAT ACA CTC CTT AAT TGA
7201 AGA ATC GCA AAA CCA GCA AGA AAA GAA TGA ACA AGA ATT ATT GGA ATT AGA TAA ATG GGC
7261 AAG TTT GTG GAA TTG GTT TAA CAT AAC AAA TTG GCT GTG GTA TAT AAA ATT ATT CAT AAT
7321 GAT AGT AGG AGG CTT GGT AGG TTT AAG AAT AGT TTT TGC TGT ACT TTC TAT AGT GAA TAG
7381 AGT TAG GCA GGG ATA TTC ACC ATT ATC GTT TCA GAC CCA CCT CCC AAC CCC GAG GGG ACC
7441 CGA CAG GCC CGA AGG AAT AGA AGA AGA AGG TGG AGA GAG AGA CAG AGA CAG ATC CAT TCG
7501 ATT AGT GAA CGG ATC TCG ACG GTC GCC AAA TGG CAG TAT TCA TCC ACA ATT TTA AAA GAA
7561 AAG GGG GGA TTG GGG GGT ACA GTG CAG GGG AAA GAA TAG TAG ACA TAA TAG CAA CAG ACA

```
7621 TAC AAA CTA AAG AAT TAC AAA AAC AAA TTA CAA AAA TTC AAA ATT TTC GGG TTT ATT ACA
7681 GGG ACA GCA GAG ATC CAG TTT GGA TCG ATA AGC TTG ATA TCG AAT TCC TGC AGC CCC GAT
7741 AAA ATA AAA GAT TTT ATT TAG TCT CCA GAA AAA GGG GGG AAT GAA AGA CCC CAC CTG TAG
7801 GTT TGG CAA GCT AGC TGC AGT AAC GCC ATT TTG CAA GGC ATG GAA AAA TAC CAA ACC AAG
7861 AAT AGA GAA GTT CAG ATC AAG GGC GGG TAC ATG AAA ATA GCT AAC GTT GGG CCA AAC AGG
7921 ATA TCT GCG GTG AGC AGT TTC GGC CCC GGC CCG GGG CCA AGA ACA GAT GGT CAC CGC AGT
7981 TTC GGC CCC GGC CCG AGG CCA AGA ACA GAT GGT CCC CAG ATA TGG CCC AAC CCT CAG CAG
8041 TTT CTT AAG ACC CAT CAG ATG TTT CCA GGC TCC CCC AAG GAC CTG AAA TGA CCC TGC GCC
8101 TTA TTT GAA TTA ACC AAT CAG CCT GCT TCT CGC TTC TGT TCG CGC GCT TCT GCT TCC CGA
8161 GCT CTA TAA AAG AGC TCA CAA CCC CTC ACT CGG CGC GCC AGT CCT CCG ACA GAC TGA GTC
8221 GCC CGG GGG GGA TCC GTC CTC CGG CCT CGG CTG CGT CGC GCC ATG GCG GCC CCC GGC GCC
                                                          M   A   A   P   G   A
8281 CGG CGG CCG CTG CTC CTG CTG CTG CTG GCA GGC CTT GCA CAT GGC GCC TCA GCA CTC TTT
      R   R   P   L   L   L   L   L   L   A   G   L   A   H   G   A   S   A   L   F
8341 GAG AAT TCG ATG GTC AGC AAG GGC GAG GAG CTG TTC ACC GGG GTG GTG CCC ATC CTG GTC
      E   N   S   M   V   S   K   G   E   E   L   F   T   G   V   V   P   I   L   V
8401 GAG CTG GAC GGC GAC GTA AAC GGC CAC AAG TTC AGC GTG TCC GGC GAG GGC GAG GGC GAT
      E   L   D   G   D   V   N   G   H   K   F   S   V   S   G   E   G   E   G   D
8461 GCC ACC TAC GGC AAG CTG ACC CTG AAG TTC ATC TGC ACC ACC GGC AAG CTG CCC GTG CCC
      A   T   Y   G   K   L   T   L   K   F   I   C   T   T   G   K   L   P   V   P
8521 TGG CCC ACC CTC GTG ACC ACC CTG ACC TAC GGC GTG CAG TGC TTC AGC CGC TAC CCC GAC
      W   P   T   L   V   T   T   L   T   Y   G   V   Q   C   F   S   R   Y   P   D
8581 CAC ATG AAG CAG CAC GAC TTC TTC AAG TCC GCC ATG CCC GAA GGC TAC GTC CAG GAG CGC
      H   M   K   Q   H   D   F   F   K   S   A   M   P   E   G   Y   V   Q   E   R
8641 ACC ATC TTC TTC AAG GAC GAC GGC AAC TAC AAG ACC CGC GCC GAG GTG AAG TTC GAG GGC
      T   I   F   F   K   D   D   G   N   Y   K   T   R   A   E   V   K   F   E   G
8701 GAC ACC CTG GTG AAC CGC ATC GAG CTG AAG GGC ATC GAC TTC AAG GAG GAC GGC AAC ATC
      D   T   L   V   N   R   I   E   L   K   G   I   D   F   K   E   D   G   N   I
8761 CTG GGG CAC AAG CTG GAG TAC AAC TAC AAC AGC CAC AAC GTC TAT ATC ATG GCC GAC AAG
      L   G   H   K   L   E   Y   N   Y   N   S   H   N   V   Y   I   M   A   D   K
8821 CAG AAG AAC GGC ATC AAG GTG AAC TTC AAG ATC CGC CAC AAC ATC GAG GAC GGC AGC GTG
      Q   K   N   G   I   K   V   N   F   K   I   R   H   N   I   E   D   G   S   V
8881 CAG CTC GCC GAC CAC TAC CAG CAG AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG CCC
      Q   L   A   D   H   Y   Q   Q   N   T   P   I   G   D   G   P   V   L   L   P
8941 GAC AAC CAC TAC CTG AGC ACC CAG TCC GCC CTG AGC AAA GAC CCC AAC GAG AAG CGC GAT
      D   N   H   Y   L   S   T   Q   S   A   L   S   K   D   P   N   E   K   R   D
9001 CAC ATG GTC CTG CTG GAG TTC GTG ACC GCC GCC GGG ATC ACT CTC GGC ATG GAC GAG CTG
      H   M   V   L   L   E   F   V   T   A   A   G   I   T   L   G   M   D   E   L
9061 TAC AAG CTT CGC ATG AAG GGA TCC GGC ATC GCT GGC TTC AAA GGT GAA CAA GGC CCC AAG
      Y   K   L   R   M   K   G   S   G   I   A   G   F   K   G   E   Q   G   P   K
9121 GGA GAA CCT TCA TGG GAG AAT CTG AAG GGG TCT GTG GAA GAG TGT GTT CAG GAT GGT AAC
      G   E   P   S   W   E   N   L   K   G   S   V   E   E   C   V   Q   D   G   N
9181 AAC ATG TTG ATC CCC ATT GCT GTG GGC GGT GCC CTG GCA GGG CTG GTC CTC ATC GTC CTC
      N   M   L   I   P   I   A   V   G   G   A   L   A   G   L   V   L   I   V   L
9241 ATT GCC TAC CTC ATT GGC AGG AAG AGG AGT CAC GCC GGC TAT CAG ACC ATC TAG CTC GAG
      I   A   Y   L   I   G   R   K   R   S   H   A   G   Y   Q   T   I   Z
9301 AGG CCT GGT ACC ACG CGT GCG GCC GCG ACT CTA GAG TCG ACC TGC AGG CAT GCA AGC TTG
9361 ATA TCA AGC TTA TCG ATG AAT GTC AAT AAA ACA AAA TGG TGG GGC AAT CAT CTA CAT TTC
9421 ATG GGA TAT GTG ATT ACT AGT TCA GGT GTA TTG CCA CAA GAC AAA CAT GTT AAG AAA ATT
9481 TCC CGT TAT TTG CAC TCT GTT CCT GTT AAT CAA CCT CTG GAT TAC AAA ATT TGT GAA AGA
9541 TTG ACT GGT ATT CTT AAC TAT GTT GCT CCT TTT ACG CTA TGT GGA TAC GCT GCT TTA ATG
9601 CCT TTG TAT CAT GCT ATT GCT TCC CGT ATG GCT TTC ATT TTC TCC TCC TTG TAT AAA TCC
9661 TGG TTG CTG TCT CTT TAT GAG GAG TTG TGG CCC GTT GTC AGG CAA CGT GGC GTG GTG TGC
9721 ACT GTG TTT GCT GAC GCA ACC CCC ACT GGT TGG GGC ATT GCC ACC ACC TGT CAG CTC CTT
9781 TCC GGG ACT TTC GCT TTC CCC CTC CCT ATT GCC ACG GCG GAA CTC ATC GCC GCC TGC CTT
9841 GCC CGC TGC TGG ACA GGG GCT CGG CTG TTG GGC ACT GAC AAT TCC GTG GTG TTG TCG GGG
9901 AAG CTG ACG TCC TTT CCA TGG CTG CTC GCC TGT GTT GCC ACC TGG ATT CTG CGC GGG ACG
9961 TCC TTC TGC TAC GTC CCT TCG GCC CTC AAT CCA GCG GAC CTT CCT TCC CGC GGC CTG CTG
10021 CCG GCT CTG CGG CCT CTT CCG CGT CTT CGC CTT CGC CCT CAG ACG AGT CGG ATC TCC CTT
10081 TGG GCC GCC TCC CCG CCT GAT CGA TAC CGT CGA CCT CGA
```

Art is never finished, only abandoned.
- Leonardo da Vinci